MAR 1 8 1947

Official Organ of the Society for the Study of Development and Growth

GROWTH

A JOURNAL FOR STUDIES OF DEVELOPMENT AND INCREASE

VOLUME X, NUMBER 4
PAPERS 304-308

DECEMBER, 1946 **$5.00 A YEAR**

Entered as second class matter on October 20, 1941, at the Post Office, Worcester, Mass., Under the Act of March 3, 1879.

GROWTH

A journal for the coördination of studies of increase and development as general properties of nature. Published by contributors and subscribers on a coöperative, non-profit, non-salary basis. The field of investigation is primary and work will be published in which the approach is through mathematics, physics, chemistry, genetics, embryology, philosophy, and anthropology; just as will studies of development in its several expressions, and of mass increase and the part played therein by nutrition, vitamines, and hormones.

The journal will appear regularly at quarterly intervals. Subscription is $5.00 per volume (outside U. S. and Canada, $5.50). Single numbers $2.00. There will be one volume of at least 400 pages each year.

Subscriptions, manuscripts submitted for publication, and all correspondence should be sent to *The Editors of Growth, Lankenau Hospital Research Institute, Girard and Corinthian Avenues, Philadelphia 30, Pa.*

THE EDITORS OF GROWTH

John Berrill, McGill University; S. Brody, University of Missouri; H. S. Burr, Yale University; S. A. Courtis, University of Michigan; C. H. Danforth, Stanford University; L. K. Frank, Macy Foundation; P. W. Gregory, University of California; F. S. Hammett, Lankenau Hospital; Leigh Hoadley, Harvard University; Clyde Kluckhohn, Harvard University; Warren H. Lewis, The Wistar Institute; Leo Loeb, Washington University; Carroll E. Palmer, U. S. Public Health Service; Otto Rahn, Cornell University; H. S. Reed, University of California; S. P. Reimann, Lankenau Hospital; R. E. Scammon, University of Minnesota; E. W. Sinnott, Yale University; K. V. Thimann, Harvard University; Paul Weiss, University of Chicago; Philip R. White, Rockefeller Institute; D. M. Whitaker, Stanford University; B. H. Willier, Johns Hopkins University; Sewall Wright, University of Chicago.

Corresponding Editors

B. Boysen Jensen, University of Copenhagen; Ivar Broman, Tornblad Institutet; Luigi Castaldi, Università di Cagliari; A. Dalcq, Université de Bruxelles; E. Fauré-Fremiet, College de France; Julian S. Huxley, Zoölogical Society; J. Krizenecky, Vysoka Skola Zemedelska; Joseph Needham, Caius College; Yo. K. Okada, Kyoto Imperial University; G. Teissier, Faculté des Sciences; A. H. Roffo, Universidad de Buenos Aires.

Growth, 1946, **10**, 343-359

ACTION DE LA LUMIÈRE ET DE LA TEMPERATURE SUR LA CROISSANCE ET LA SEXUALITÉ DES MALES ET DES FEMELLES DE *MERCURIALIS ANNUA L.*

GEORGES TROUPIN

Laboratoire de Physiologie Végétale, Institut de Botanique de l'Université de Liége

(Received for publication September 15, 1946)

INTRODUCTION

La question de la sexualité des plantes présente une réelle importance tant théorique que pratique.

L'on conçoit aisément l'interêt économique qui s'attache à la production des fruits; mais les recherches réalisées dans ce domaine ne manquent pas d'intérêt spéculatif. On peut en effet aborder l'étude de la sexualité par des méthodes cytologiques ou physiologiques, et tout d'abord y distinguer plusieurs aspects: (a) l'apparition de la sexualité, (b) le déterminisme du sexe, (c) les caractéristiques des sexes.

Chacun de ces aspects peut être traité et a été traité ou bien à l'échelle cellulaire, ou encore dans le plan de la morphogenèse, ou enfin dans le jeu du métabolisme et des hormones.

Le but du présent travail est une étude préliminaire des phénomènes de sexualité envisagée du point de vue physiologique. Cette recherche s'intègre dans un ensemble de travaux réalisés dans divers laboratoires étrangers et dans le laboratoire de Physiologie végétale de l'Université de Liège (Direct. Prof. R. BOUILLENNE).

Nous avons choisi comme matériel *Mercurialis annua L.* (Euphorbiacées). Cette plante accomplit son cycle vital en quelques mois ce qui nous permet de multiplier nos séries d'observations. Par son caractère dioïque, les mâles et les femelles peuvent être étudiés séparément.

M. BOUILLENNE-WALRAND et R. BOUILLENNE (3)* se sont attachés aux rapports entre la différenciation des sexes et le métabolisme. Ils ont montré que les mâles en pleine floraison se caractérisent par

*Les chiffres entre parentheses renvoient à la littérature citée p. 358.

un contenu en glucides et un métabolisme respiratoire plus élevés que les femelles au même stade. L. GHENNE (8) a suivi les variations dans le cycle vital des plantes mâles et des plantes femelles de *Mercurialis perennis,* s'attachant principalement aux fluctuations du contenu en glucides des rhizomes. Divers auteurs, LAURENT (11), SOURVILLE (13), GILLOT (9) et FOUARGE (5) ont présenté sur ce sujet de nombreuses observations malheureusement non concordantes. Ce fait es dû, nous semble-t-il, à ce que les auteurs ne précisaient pas suffisamment les stades auxquels se trouvaient les individus examinés ou expérimentés.

Il serait donc nécessaire, avant tout autre travail, de préciser soigneusement les étapes successives par lesquelles passent les individus de l'espèce que nous avons choisie au cours de leur existence à partir de la germination. Pour fixer ces étapes, il faut envisager les caractéristiques de la croissance et du développement. (LYSENKO—12) "La croissance est un *phénomène* quantitatif d'accroissement de taille et de poids; le développement est *un phénomene qualitatif* caractérisé par une série de réajustements biochimiques internes que la plante entreprend au cours de son ontogenèse pour aboutir à la reproduction sexuée." (1)

Nous avons donc examiné très minutieusement la croissance et le développement de nombreux individus de *Mercurialis annua L.*

TECHNIQUE

Pour toutes nos expériences, nous avons employé les graines provenant d'un unique et vaste semis, réalisé à cet effet au mois de mars 1945. Nous avons pu ainsi récolter vers la fin mai une quantité considérable de graines obtenues toutes dans les mêmes conditions. Ces graines, triées, de même grosseur, ont été semées un mois plus tard, sans préparations spéciales, dans des caisses contenant du terreau, composé moitié d'humus et moitié de terre de jardin.

Nous avons constaté lors du premier semis que la germination était tres capricieuse. Aussi, nous n'avons utilisé que la première levée de graines, éliminant toutes les plantules apparaissant ultérieurement.

Les semis ont été réalisés ensuite dans diverses conditions: à l'extérieur, en serre, en cave et en chambre thermostatique avec des durées d'éclairement journalier et des températures différentes. Chaque condition sera précisée au cours de l'exposé.

Nous avons suivi attentivement la marche de ces cultures. A chaque

étape apparaissant dans le cycle biologique des plantes, nous avons prélevé des individus qui exprimaient par leur aspect morphologique et leurs divers caractéristiques l'état moyen du plus grand nombre de plantes. Les échantillons sont donc aussi semblables que possible. Ils ont même âge chronologique, même hauteur, meme nombre de noeuds, d'entrenoeuds, de feuilles, même aspect.

Cette mêthode basée sur le choix rigoureux d'un petit nombre d'exemplaires nous semble préférable à celle qui consiste à prendre au hasard un lot considérable d'individus sans distinction d'âge ou d'aspect.

L'examen morphologique et les mensurations des plantes ont été faits tous les huit jours.

Resultats

A. *Conditions Normales.* Examinons tout d'abord la croissance et le développement de *Mercurialis annua L.* dans les conditions normales extérieures des mois d'été 1945.

L'apparition des boutons floraux a lieu lorsque la plante présente encore un aspect juvénile (21 jours aprés le semis, fig. 1) les plantules montrent outre leurs deux cotylédons, une première paire de feuilles commençant à s'étaler et une seconde enroulée et minuscule. A l'aisselle de la première paire de feuilles apparaissent des boutons de deux sortes sur pieds différents: les uns ont l'apparence d'une tête d'épingle subdivisée en cercles microscopiques—ce sont les boutons mâles; les autres ont la forme de petits plateaux discoïdaux—ce sont les boutons femelles. Seul, ce dimorphisme sexuel, invisible sur la Fig. 1, permet de distinguer les mâles des femelles, car l'aspect de l'appareil végétatif est semblable chez les individus des deux sexes.

Cette observation nous permet de rejeter les résultats antérieurs qui mettaient en évidence un dimorphisme sexuel secondaire (taille, ramifications, épaisseur de la tige) avant l'apparition du dimorphisme sexuel primaire (fleurs).

Les boutons floraux apparaissent successivement dès qu'une nouvelle paire de feuilles commence à s'étaler. Il s'en suit qu'il ne suffit pas de dire que *Mercurialis annua* est en fleurs, il faut préciser à quel noeud les observations sur les phénomènes sexuals sont faites. En effet, dans le cours normal de la croissance, l'anthèse des fleurs femelles se réalise environ huit jours après l'apparition des boutons femelles; l'anthèse des fleurs mâles quinze jours après l'apparition

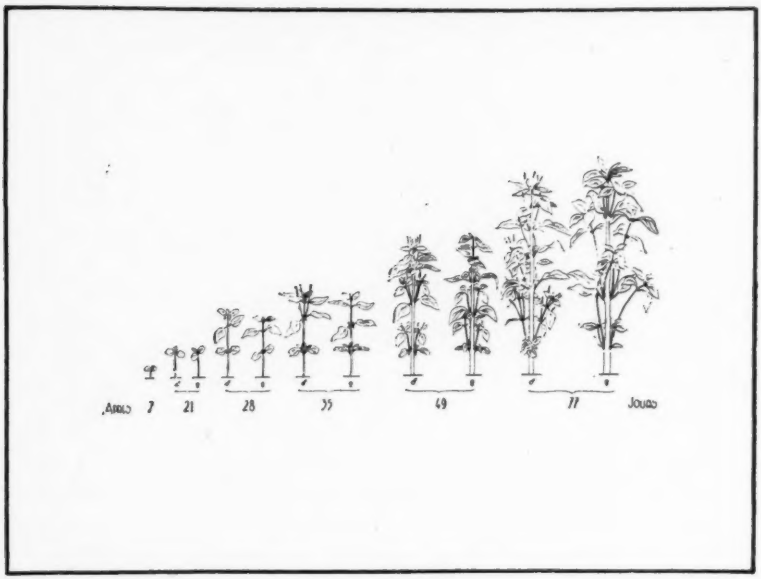

FIGURE 1
Aspect morphologique des plantes mâles et des plantes femelles à différentes étapes de leur croissance.

des boutons mâles et les graines sont formées vers le 21-ieme jour après l'anthèse des fleurs femelles (fig. 1). C'est ainsi que nous pouvons trouver des plantes qui à certains stades de croissance montrent à différents noeuds toutes les étapes de la sexualité. Dans ce présent travail, nous nous attachons aux premières manifestations extérieures de la sexualité, c'est-à-dire celles qui se réalisent au premier noeud foliare.

Si dans la figure 1, nous examinons les tailles des individus de chaque sexe, nous voyons que le 21e jour il n'y a pas de différences; après 28 jours, les mâles sont légèrement plus grands que les femelles; après 35 jours les deux sexes sont approximativement la même taille; plus tard, les femelles sont plus grandes que les mâles.

Ceci nous amène à examiner l'allure générale des courbes de croissance chez les mâles et chez les femelles (fig. 2). La courbe de crois-

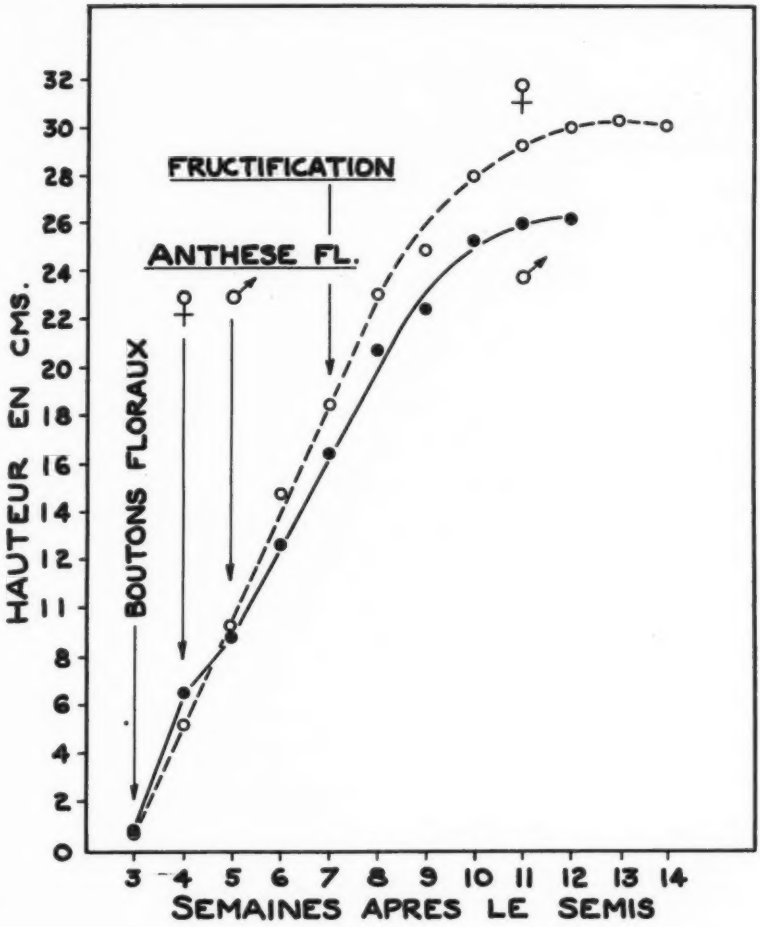

FIGURE 2

Courbe de croissance en hauteurs totales des tiges mâles et des tiges femelles de *Mercurialis annua* dans les conditions de culture normales extérieures des mois de juin à septembre 1945.

sance en hauteurs totales, construite sur un diagramme ayant en abscisses le temps exprimé en jours et en ordonnées les hauteurs exprimées en cms, nous montre qu'au début de la croissance, c'est-à-dire vers le 28e jour, les mâles sont plus grands que les femelles. Plus tard, il y a un changement dans l'allure de la courbe des plantes mâles, la croissance des mâles est ralentie tandis que celle des femelles continue au même rythme. Il en résulte que les femelles atteignent finalement une taille supérieure à celle des mâles. Disons tout de suite que nous retrouverons ce même phénomène dans toutes les courbes de croissance, quelles que soient les conditions de culture.

Ce diagramme explique clairement les divergences de vue des auteurs qui décrivent les mâles tantôt comme plus grands, tantôt comme plus petits que les femelles. Ils n'ont pas précisé à quel moment de la croissance ils avaient fait leurs observations. Ce résultat peut s'exprimer aussi en mettant en diagramme les vitesses de croissance des divers entrenoeuds des mâles et des femelles? Dans la figure 3, nous avons indiqué en abscisses les entrenoeuds suivant leur ordre d'apparition sur la plante et en ordonnées les vitesses de croissance de ces entrenoeuds exprimées en mm. par jour. On voit très clairement que la vitesse d'élongation du premier entrenoeud est plus grande chez les mâles que chez les femelles; ce qui produit la taille que nous avons toujours observée plus grande chez les mâles que chez les femelles aux premiers stades de croissance. D'un autre côté, les vitesses de croissance des autres entrenoeuds sont supérieures chez les femelles; ce qui correspond chez celles-ci à une taille finale plus grande que chez les mâles.

Ce que nous avons constaté dans la courbe des hauteurs totales, nous le retrouvons dans l'étude de la courbe des poids secs (fig. 4).

On constate aussi que le poids sec des mâles est d'abord supérieur à celui des femelles pour devenir dans la suite inférieur à celui des femelles.

En résumé, de ces premières expériences, nous dirons donc:

1°. que les boutons floraux apparaissent *d'abord* au premier noeud.

2°. que ces boutons chez les mâles et chez les femelles sont visibles 21 jours en moyenne aprés le semis.

3°. qu'avant l'anthèse des fleurs mâles au premier noeud, la taille et le poids sec des plantes mâles sont supérieurs à ceux des plantes femelles.

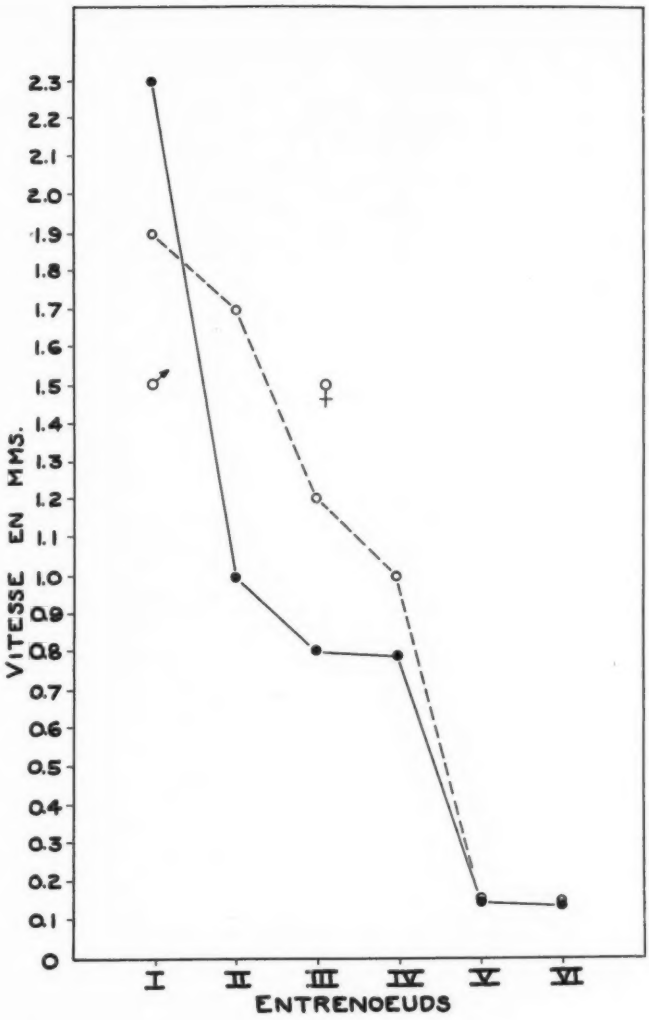

FIGURE 3

Vitesse d'élongation des différents entrenoeuds des plantes mâles et des plantes femelles de *Mercurialis annua*.

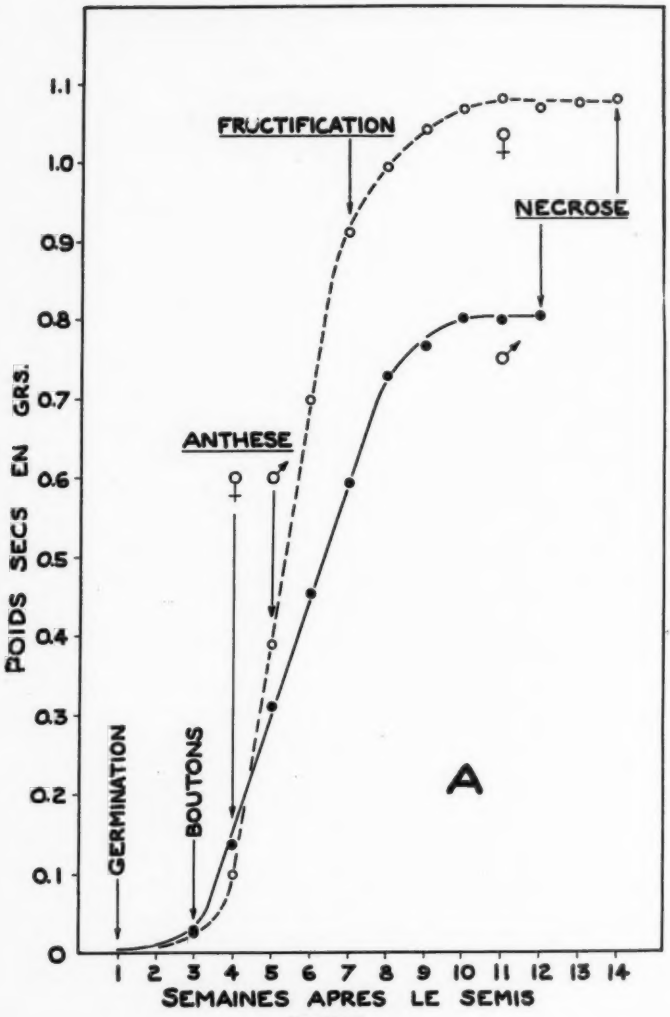

FIGURE 4

Courbe de croissance, exprimée en poids secs, des mâles et des femelles de *Mercurialis annua* dans les conditions de culture normales extérieures des mois de juin à septembre 1945.

4°. que pour finir les plantes mâles se distinguent des plantes femelles par une hauteur et un poids sec plus petits.

5°. que les plantes femelles vivent plus longtemps que les plantes mâles.

B. *Conditions Modifiées.* La deuxime série d'expériences va essayer de déterminer si une modification des conditions externes n'amènerait pas une transformation dans l'allure de ces phénomènes. Parmi les facteurs externes, nous avons choisi la lumière et la température parce que ce sont les facteurs qui se sont révélés généralement les plus importants dans l'étude de la croissance et des phases de développement.

Nous avons donc réalisé des séries d'expériences où les plantes étaient soumises à des conditions variables de lumière et de température. Ces conditions et les résultats généraux sont résumés dans le tableau I. Nous avons ainsi réalisé quatre séries différentes par les conditions de culture et par les durées de lumière et les températures utilisées. A l'intérier de chaque série, nous avons soumis les plantes à des durées d'éclairement variables allant de 8 à 20 heures par jour. En outre, pour certaines séries, nous avons contrôlé la température pendant la période d'obscurité.

Le premier résultat qui saute aux yeux est que les variations des conditions expérimentales n'ont amené aucune modification dans la localisation des premiers boutons floraux: *dans toutes les séries, ces boutons restent localisés au premier noeud.* Il n'y a jamais eu de boutons floraux au noeud cotylédonnaire et d'autre part le premier noeud n'a jamais été stérile. Le tableau montre que dans une série (serre et chambre thermostatique) on obtient les boutons floraux 12 jours en moyenne après le semis, ce qui constitue un gain de 9 jours sur les plantes croissant à l'extérieur et un gain de 5 jours sur les plantes croissant en serre.

Ce résultat est-il dû à l'action de la lumière?

L'examen du tableau montre que, à l'intérieur de chaque série, les boutons floraux sont apparus en moyenne après le même nombre de jours quelle que soit la durée d'éclarement qui variait de 8 à 20 heures.

Nous pouvons donc conclure que la durée d'éclairement n'a pas modifié le moment d'apparition des boutons floraux au premier noeud.

Maintenant comparons les séries entre elles. Nous voyons immédiatement l'influence capitale de la température et nous pouvons même

préciser que cette action s'exerce *surtout pendant la période d'obscurité*

Comparons 3 séries semblables, c'est-à-dire les séries II, III et IV (tableau I). Nous voyons que pendant la période d'éclairement, la température est approximativement la même partout. Au contraire, les températures sont trés différentes pendant les périodes d'obscurité.

On constate que lorsque la température est de 10° C. pendant la période d'obscurité, les premiers boutons floraux n'apparaissent que 26 jours aprés le semis, c'est-à-dire 9 jours plus tard que lorsque cette température est de 14° C.. Au contraire quand la température est de 23° C. pendant la période d'obscurité, les boutons floraux apparaissent aprés 12 jours soit respectivement 5 jours et 14 jours plus tôt que dans les séries avec les températures nocturnes de 14° C. et de 10° C..

Dans la série, serre et chambre thermostatique (série IV), nous constatons en outre que la température élevée pendant la période d'obscurité réduit le nombre de jours à 12 lorsque sa *durée d'action est au moins de 6 heures*. Si la température nocturne n'agit que pendant 4 heures, on obtient un chiffre qui se rapproche de la série II.

Voyons maintenant si la modification des facteurs externes amène des perturbations dans les courbes de croissance exprimée en taille et en poids sec chez les mâles et chez les femelles.

Nous avons réalisé l'étude de ces courbes de croissance dans 5 conditions indiquées dans le tableau II. Les lettres A.B.C.D.E. des diverses séries sont reprises dans les diagrammes de la fig. 5 (croissance en hauteurs totales).

On constate que les diverses conditions n'ont pas modifié l'allure générale des courbes de croissance. On retrouve dans chaque série que les mâles sont d'abord plus grand que les femelles et qu'après l'anthèse des fleurs mâles, les femelles sont au contraire plus grandes que les mâles; elles atteignent une taille finale plus élevée et elles vivent plus longtemps.

Cette persistance des caractéristiques respectives des mâles et des femelles s'observe également dans les mesures de poids secs.

Nous n'entrerons pas ici dans le détail de l'analyse de ces courbes qui montrent encore d'autres points intéressants comme, par exemple, leur rapprochement ou leur écartement.

TABLEAU I.

APPARITION ET LOCALISATION DES PREMIERS BOUTONS FLORAUX SOUS DIFFERENTES CONDITIONS DE TEMPERATURE ET DE LUMIÈRE

CONDITIONS EXPERIMENTALES A PARTIR DU SEMIS						RESULTATS		
Series	Conditions de Culture	Heures D'Eclairement Quotidien	Heures D'Obscurité Quotidienne	Temperature Diurne	Temperature Nocturne	Localisation Des Premiers Boutons Floraux	Nombre de Jours Pour L'Apparition des 1e Boutons Floraux	
I	Exterieur	17	7	21°6	11°8	1e noeud	21	21
		14	10				21	
		12	12				21	
		10	14				21	
		9	15				21	
		8	16				21	
II	Serre	20	4	26°4	14°3	1e noeud	17	17
		18	6				17	
		14	10				17	
		12	12				17	
		10	14				18	
		8	16				17	
III	Serre (lumière) et Cave (obscurité)	20	4	24°3	10°	1e noeud	26	26
		18	6				25	
		14	10				27	
		10	12				26	
IV	Serre (lumière) et Chambre Thermostatique (obscurité)	20	4	24°3	23°	1e noeud	15	15
		18	**6**				**12**	12
		14	10				12	
		10	12				12	

TABLEAU II.

TAILLES FINALES ET POIDS SECS FINAUX OBTENUS SOUS DIFFERENTES CONDITIONS DE TEMPERATURE ET DE LUMIÈRE

Series	Conditions de Culture	Heures D'Eclairement Quotidien	Heures D'Obscurité Quotidienne	Temperature Diurne	Temperature Nocturne	Tailles Finales en Cms.		Poids Secs Finaux en Grs.	
						♂	♀	♂	♀
A	Exterieur	14-17	7-10	21°6	11°8	26.4	30.4	0.8112	1.0847
B	Exterieur	9	15	21°6	11°8	20.5	22.1	0.3602	0.6267
C	Serre	14-17	7-10	26°4	14°3	16.5	19.8	0.3597	0.4910
D	Serre	12-14	10-12	29°3	17°2	20.6	23.6	0.3281	0.5419
E	Serre (lumière) et Chambre Thermostatique (obscurité)	18	6	24°3	23°	22.8	24.7	0.3402	0.4756

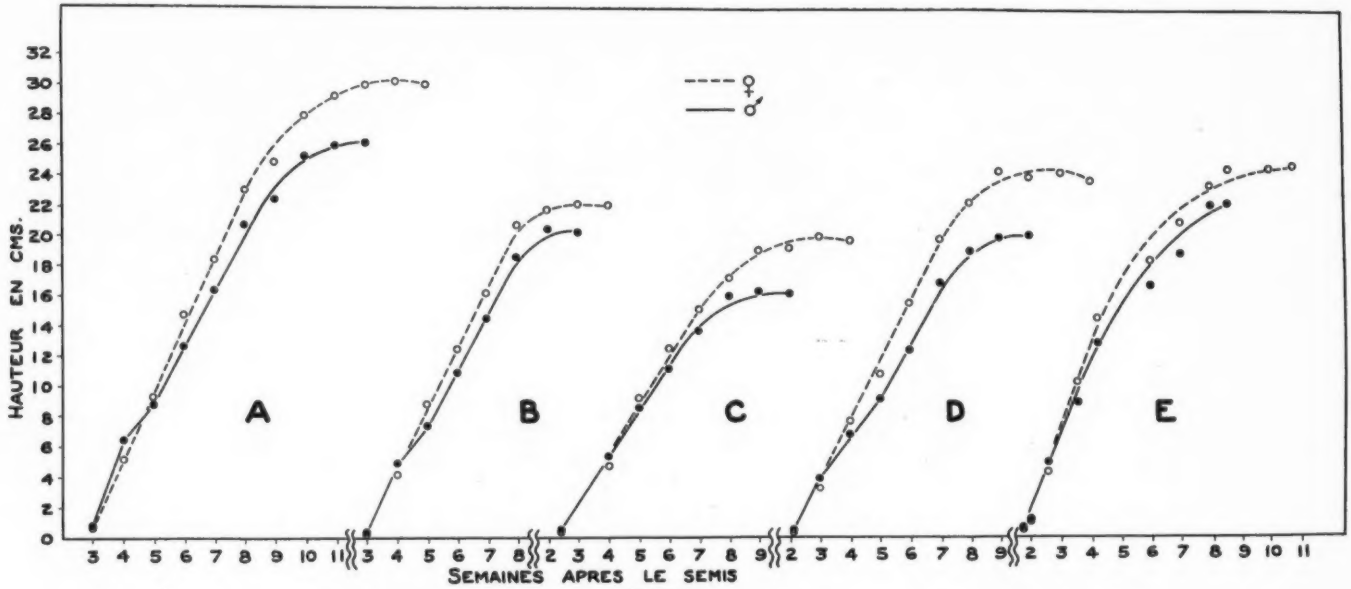

FIGURE 5

Courbes de croissance en hauteurs totales (mesurées à partir du noeud cotylédonnaire) des tiges des mâles et des femelles de *Mercurialis annua* sous differentes conditions de culture. Pour le détail de ces conditions (A, B, C, D, E) voir le tableau II.

Discussion

Nos expériences montrent que le dimorphisme sexuel primaire chez les plantes mâles et chez les plantes femelles de *Mercurialis annua* s'accompagne d'un dimorphisme sexuel secondaire. Les mâles commencent à se distinguer par une taille légèrement plus grande que les femelles, puis, ils ont une taille finale moins élevée, des ramifications latérales moins abondantes, une couleur verte de feuilles plus pâle et un poids sec final plus petit que chez les femelles. De plus les mâles ont une vie plus courte que celles des femelles. Comme R. Bouillenne et Mme. Bouillenne-Walrand l'ont montré, ces différences doivent être mises en rapport avec un métabolisme différent chez les mâles et chez les femelles.

Le fait que ce dimorphisme sexuel secondaire ne se manifeste qu' après l'anthèse des fleurs et que les caractères sexuels primaires apparaissent à un stade très juvénile des plantules, semble indiquer, pensons-nous, que les caractères sexuels secondaires chez *Mercurialis annua* se trouveraient sous la dépendance des caractères sexuels primaires.

Cette conception est confirmée par le fait que les différences profondes dans les conditions de culture n'ont pas amené de modifications importantes dans l'allure des courbes générales de croissance des mâles et des femelles. L'allure de ces courbes de croissance semble être sous la dépendance de facteurs internes auxquels nos conditions expérimentales n'ont pas touché.

De plus, l'accélération dans le temps d'apparition des boutons floraux nous paraît être due à une action des facteurs externes sur la croissance de *Mercurialis annua L.* et non sur son développement, car les premiers boutons floraux, quelles que soient les conditions, restent strictement localisés au premier noeud.

Nos résultats mettent en évidence un point très important: l'action accélératrice de la température pendant la période d'obscurité sur le moment d'apparition des fleurs.

Cette action, qui doit au moins durer 6 heures, indique aussi que nous avons agi sur la croissance, et vraisemblablement sur la croissance par multiplication cellulaire, car l'action de la température n'est décisive que pendant la période d'obscurité, moment où l'on sait que les mitoses cellulaires sont les plus intenses chez les végétaux.

Ces conclusions nous conduisent à critiquer la subdivision des plantes en plantes à longs jours, plantes à courts jours et plantes

indifférentes, telle qu'elle a été présentée par GARNER et ALLARD (6 et 7) et telle qu'elle est utilisée encore à l'heure actuelle dans de nombreux travaux traitant du photopériodisme chez les végétaux. Pour ces auteurs, les plantes à longs jours sont celles où le moment de la floraison est hâté quand on augmente la durée d'éclairement journalier. Les plantes à courts jours, au contraire fleurissent moins rapidement si la durée d'éclairement journalier est trop prolongée. Les plantes indifférentes sont celles qui, dans de larges limites, sont insensibles pour leur floraison à la durée d'éclairement.

Comme nous l'avons dit, nos expériences montrent que la température pendant la période d'obscurité diminue le délai d'apparition des fleurs et que cela semble dû à une action sur la croissance: nous n'avons pas touché au développement.

GARNER et ALLARD ainsi que de nombreux auteurs de ces derniéres années n'établissent pas de maniére claire la valeur de leurs résultats. Nulle part dans leurs travaux, on ne trouve de précisions sur la localisation des fleurs lorsque l'apparition de celles-ci est hâtée par l'action d'un certain photopériodisme. Ainsi par exemple, nous ne savons pas si dans leurs expériences ils ont réussi à faire apparaitre des fleurs au 5e noeud foliaire d'une plante qui normalement n'en montre qu'au 10e, ce qui prouverait qu'ils ont agi sur le développement.

Il nous semble qu'il conviendrait de réagir contre cette confusion encore trop fréquente. Pour cela, suivant l'exemple de CAJLAHJAN (4), BONNER et HAMNER (10) qui ont réussi à mettre en évidence la présence d'hormones de floraison chez les végétaux, nous proposons de réserver le nom de "plantes à longs jours" aux plantes qui ne fleurissent pas si la durée d'éclairement journalier est insuffisante et le nom de "plantes à jours court" aux plantes qui ne fleurissent pas si elles sont soumises à une durée d'éclairement journalier trop prolongée.

RÉSUMÉ

Ce travail nous a permis de soulever quelques questions qui nous semblent intéressantes dans les problèmes posés par le cycle vital des mâles et des femelles de *Mercurialis annua:*

1) Il y a une évolution différencielle des courbes de taille et de poids secs des plantes mâles et des plantes femelles, courbes qui changent d'allure à partir du moment de la formation des boutons floraux;

2) Les mâles sont plus grands et pèsent plus que les femelles au début de la floraison;

3) Les mâles diminuent leur vitesse de croissance à partir de l'anthèse de leurs fleurs (vers le 35e jour après le semis). Vers le même moment, le poids sec des plantes femelles devient plus élevé que celui des plantes mâles. Ce caractère se maintient jusqu'à la fin de la végétation.

4) L'avantage temporaire des mâles est en coincidence avec les caractéristiques du 1r entrenoeud de la tige.

5) Les conditions extérieures de culture ont été modifiées systématiquement en ce qui concerne surtout la température et la durée d'éclairement.

6) Ces conditions n'ont pas modifiés le régime de la maturation sexuelle des mâles et des femelles qui a toujours été réalisé sur le premier noeud de la tige.

7) Elles ont modifié la vitesse de l'apparition des boutons floraux dans les deux sexes.

8) Les conditions qui ont diminué de 21 jours à 12 jours le temps nécessaire à la formation des boutons floraux sont une température élevée administrée durant la période d'obscurité pendant au moins 6 heures.

9) Les plantes femelles ont une longévité plus grande que les plantes mâles.

Je tiens à remercier vivement Monsieur le Professeur R. BOUILLENNE qui s'est intéressé très activement à l'élaboration de ce travail.

LITTÉRATURE CITÉE

1. BOUILLENNE, R. 1946. Cours de Phytobiologie.
2. BOUILLENNE, R. & M. 1930. La sexualité et les oxydations cellulaires chez *Mercurialis annua*. (*Bull. Acad. royal Belg.*, **17**, p. 1047)
3. ————. 1931. Oxydations respiratoires et teneur en sucre dans les deux sexes de *Mercurialis annua*. (*Classe des Sc.*, **18**, p. 980)
4. CAJLAHJAN. 1936. On the hormonal theory of plant development. (*Doklady Ac. Sc. U. R. S. S.*, **3**, p. 442)
5. FOUARGE, M. 1931. Observations sur la croissance de *Mercurialis annua*. (*Thèse doctorat, Inst. Bot., Univ. de Liège*)
6. GARNER & ALLARD. 1920. Effect of the relative length of day and night and other factors of the environment on growth and reproduction in plants. (*Jour. Agric. Res.*, **18**, p. 353)
7. ————. 1923. Further studies in photoperiodism; the response of the plant to relative length of day and night. (*Jour. Agric. Res.*, **23**, p. 871)

8. GHENNE, L. 1933. Etude des phénomènes de la croissance et du mètabolisme différentiels chez les males et les femelles de *Mercurialis perennis*. (*Thèse doctorat, Inst. Bot., Univ. Liège*)

9. GILLOT. 1924. Remarques sur le déterminisme du sexe chez *Mercurialis annua*. (*C. R. Ac. Sc.*, **178**, p. 1995)

10. HAMNER & BONNER. 1938. Photoperiodism in relation to hormones as factors in floral initiation and development. (*Bot. Gaz.*, **100**, p. 388)

11. LAURENT. 1906. Une nouvelle hypothèse sur le déterminisme du sexe. (*C. R. Ass. Fr. Av. Sc. 35e session*, p. 413)

12. LYSENKO. 1928. *Trud. Azerbaidz. Stat.*, **5**, p. 1-168.

13. SOUVILLE. 1925. Observations sur le dimorphisme sexuel de *Mercurialis annua*. (*Rev. gén. Bot.*, **37**, p. 434)

Growth, 1946, **10**, 361-373

TOLERANCE OF LARVAE OF DROSOPHILA FOR AMINO ACIDS: METHIONINE, CYSTINE, AND CYSTEINE*

LOUISE PALMER WILSON

Wellesley College, Wellesley, Massachusetts; and the Marine Experimental Station of the Lankenau Hospital Research Institute, North Truro, Massachusetts

(Received for publication September 18, 1946)

The effects of administering the sulphur-containing amino acids, methionine, cystine, and cysteine to animals maintained on a balanced diet have been studied in only a few cases. Of the three acids, methionine is generally considered indispensible in the diet of vertebrates but under certain conditions may be partially replaced by cysteine or cystine (Almquist and Grau, 1945 and Treadwell, 1945). Methionine and not cystine is concerned in the prevention of fatty livers in mammals (Beveridge, Lucas and O'Grady, 1945). Experiments by Beams (1946) on human patients however show that cystine and choline are as effective as methionine. Excess dl-methionine and not cystine or cysteine prevents burn toxemia in rats (Croft and Peters, 1945). In dogs, cysteine HCl acts as an antidote to the toxic action of the organic mercurial diuretic, Salyrgan (Long and Farah, 1946). Diets supplemented with cystine decrease the induction period for hepatomas in rats (White and Edwards, 1942). Additional methionine will prevent inhibition of growth by norleucine in *Proteus morganii* but l-cystine has no such effect (Porter and Myers, 1945). In the coelenterate *Obelia* methionine acts as a growth stimulant for increase in number of cells, possibly by conversion to cystine and then to cysteine (Hammett and Padis, 1936) in accord with the SH hypothesis of Hammett (1929). Kotake (1934) reports that the tolerance for cystine is low in rats and that nephrosis results when extra cystine is added to the basal diet. A good general review of early work concerning the metabolism of these amino acids is given by Toennies (1937).

This paper is one of a series of reports of investigations on the tol-

*Supported in part by a grant from the Women's Auxiliary of the Lankenau Institute for the Promotion of Cancer Research.

erance of the larvae of Drosophila for protein constituents added to a basal diet. Rate of growth and ecdysis of larvae, time of formation and duration of the pupal stage, and any abnormalities of the structural characteristics of adults are observed in order to determine the effect of addition of various concentrations of a given amino acid or nucleic acid.

In the present investigation, the dl-methionine and cysteine hydrochloride were obtained from Merck and Co. while the l-cystine was prepared by the staff of the Lankenau Hospital. Three experiments under sterile controlled conditions as described by Wilson and Birch (1944) were performed at each concentration, tested. Concentrations between M/10 and M/5000 were used for each amino acid. In the case of cysteine, concentrations as low as M/100,000 were used. Since cystine is insoluble in water at the higher concentrations, suspensions at each of these higher concentrations were used for this acid. In addition tests were made in which the cystine was dissolved in 0.1N. HCl before addition to the culture medium.

Results and Discussion

*Methionine.** Drosophila larvae tolerate concentrations of methionine below M/500 with no modification of any growth process (Table 1). Concentrations higher than M/50 inhibit rate of growth in size and increase the length of each larval instar. A concentration of M/100 does not modify size to a great extent but does increase the duration of larval instars. Table 2 and Figure 1, the results of typical experiments illustrate these points. The rate of moulting, which is the index of the duration of larval instars, is shown by the percentages following the numbers of experimental animals in the tables.

Many larvae die in the two higher concentrations. The growth curve at a concentration of M/10 is similar to that in tryptophane (Wilson, 1945) and to that in alanine and phenylalanine (Wilson, 1945) but few if any larvae die when treated with these amino acids at a concentration of M/10. Among those treated with M/10 methionine approximately 80% of the eggs failed to hatch or died before pupation. Only 25% died when treated with M/25 methionine and no abnormal deaths were observed among those treated with methionine at a concentration lower than M/50.

*With the technical assistance of Miss Dorothy Thornton.

TABLE 1

AVERAGE LENGTH AND WIDTH IN MILLIMETERS OF LARVAE TREATED WITH METHIONINE

Age	Instar	Control				M/500				M/1000			
		No.	%	Length	Width	No.	%	Length	Width	No.	%	Length	Width
48	1	18	22	1.56	.25	26	35	1.58	.27	13	12	1.50	.24
	2	63	78	2.03	.28	78	65	1.97	.29	93	88	2.07	.29
72	2	23	46	2.98	.44	39	50	3.00	.47	33	44	3.02	.45
	3	27	54	3.61	.49	39	50	3.66	.52	42	56	3.62	.54
96	3	98	100	4.89	.72	75	100	4.88	.73	88	100	4.84	.71
120	3	54	95	4.84	.79	91	98	4.68	.80	63	95	4.81	.76
	P	3	5			2	2			4	5		

Age in hours from median hour of oviposition
P—pupae

TABLE 2

AVERAGE LENGTH AND WIDTH IN MILLIMETERS OF LARVAE TREATED WITH METHIONINE

		Control				M/10				M/25			
Age	Instar	No.	%	Length	Width	No.	%	Length	Width	No.	%	Length	Width
48	1	23	43	1.60	.25	35	100	1.20	.20	42	100	1.40	.23
	2	30	57	1.98	.29								
72	2	66	91	2.99	.46	34	100	1.77	.28	40	95	2.60	.42
	3	7	9	3.47	.51					2	5	3.41	.48
96	1					3	7	1.08	.16				
	2					35	81	2.08	.33	2	4	2.40	.38
	3	37	100	4.81	.74	5	12	3.06	.42	48	96	4.06	.61
120	2					3	20	2.23	.35				
	3	39	91	4.98	.80	12	80	3.36	.49	32	100	4.65	.76
		Control				M/100				M/250			
48	1	2	4	1.61	.26	3	7	1.53	.36	17	29	1.58	.25
		45	96	2.02	.29	42	93	2.01	.28	42	71	1.99	.29
72	2	9	15	3.12	.48	14	20	3.11	.46	5	13	3.03	.48
	3	51	85	3.78	.53	55	80	3.78	.52	35	87	3.76	.53
96	3	54	100	5.00	.77	61	100	4.67	.65	50	100	4.79	.70
120	3	55	98	4.65	.73	44	92	4.69	.72	51	93	4.78	.68
	P	1	2			4	8			4	7		

Age in hours from median hour of oviposition
P—pupae

The cause of death in these animals appears to be some disturbance of the moulting process. Larvae form the second or third exoskeleton while very small and then are unable to shed the old exuvia. No such observation has been made at the high concentrations of other amino acids which have been investigated. Excess methionine seems to inhibit some processes associated with development while not affecting others. There is thus an uneven development and normal moulting is often impossible. If the flies develop, they emerge apparently normal 15 days after oviposition in contrast with the nine or 10 days of the control.

In the only investigation where separate fundamental growth processes were analyzed, Hammett and Padis (1936) found that proliferation and new growth were enhanced, while differentiation and organization were unaffected and maintenance was depressed in *Obelia* treated with methionine. These same conditions much exaggerated may account for the death of the Drosophila larvae. If unorganized, undifferentiated growth occurs with no storage of potential energy, it seems likely that the elaboration of the moulting hormone would result in abnormalities.

At a concentration of M/250, methionine accelerates moulting as shown in Table 3 despite the fact that the rate of growth in length is slightly retarded (Figure 1).

TABLE 3
PER CENT OF ACCELERATION OF MOULTING IN LARVAE TREATED WITH M/250 METHIONINE

	Exp. 2	Exp. 4	Exp. 11
1st to 2nd instar	24	37	14
2nd to 3rd instar	12	7	10
3rd instar to pupa	—2	6	0

No other concentration shows such consistent acceleration. Associated with this phenomenon is a decided increase of the ratio length/width as compared with the control at 120 hours. In the three experiments the ratios were as follows:

Exp.	Control	M/250 methionine
11	1 to 6.1	1 to 6.4
4	1 to 6.0	1 to 6.6
2	1 to 6.3	1 to 6.7

These results are in contrast with those obtained from larvae treated with M/500 methionine, the results for which were as follows:

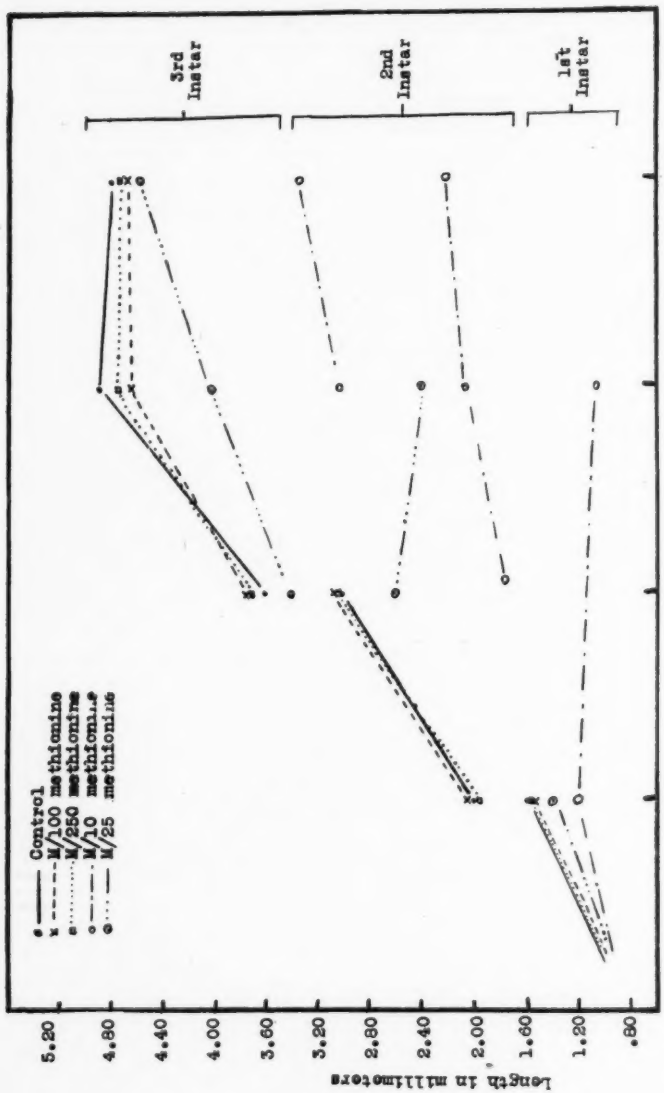

FIGURE 1

RATE OF GROWTH OF DROSOPHILA LARVAE TREATED WITH METHIONINE. Results of typical experiments are shown.

Exp.	Control	M/500 methionine
1	1 to 6.3	1 to 6.3
3	1 to 6.0	1 to 6.0
11	1 to 6.1	1 to 6.1

There is here obviously no difference between tests and controls.

These results are interesting when compared with those obtained from arginine (Wilson and Birch, 1944) where over a long effective range increased rate of moulting was associated with larger size, particularly greater width, than the control. In this case it was suggested that arginine acted as a lipogenic agent. It is possible here that methionine acts in just the opposite manner and brings about the removal of fat from the fat body. Such a result is consistent with the observations of Hammett and Padis (1936) that methionine may increase metabolic activity.

Changes in mass of Drosophila larvae may mean changes in cell size, in amount of fat deposited in the fat body or in the degree of cell division in the imaginal organ anlagen. As stated in the introduction, methionine is concerned with fat metabolism in vertebrates; it stimulates cell proliferation in *Obelia*; and it is considered an indispensible amino acid for normal growth in many organisms. Under differing conditions, therefor, methionine is reported to play some role with each of the three processes concerned with change in mass in Drosophila. Based on the present data it is pure speculation to hazard an explanation for the effects on Drosophila. It is possible that methionine accelerates moulting by stimulating proliferation of the cells producing the moulting hormone and that the energy for the process was obtained by the mobilization of fat from the fat body.

Cystine. Since l-cystine is insoluble in higher concentrations it was suspended in the basal medium in amounts equal to M/10, M/50, and M/100. The chief food of the larvae is suspended yeast cells so it is assumed that discrete particles are ingested. Even in as thick suspensions as M/10 cystine there was no evidence of toxicity nor was there any significant modification of growth processes. It is possible that the crystals pass unchanged through the gut since at no concentration from M/250 to M/10,000 was there any evidence of change in rate of growth in length or in rate of moulting. A typical experiment using M/250 cystine is shown in Table 4. At this concentration only there was evidence of modification of growth. There is a decrease of width in proportion to length. The length/width ratios at 120 hours are as follows:

Exp.	Control	M/250 cystine
3	1 to 6.3	1 to 6.7
5	1 to 6.2	1 to 6.7
7	1 to 6.5	1 to 6.7

These thinner larvae did not show an increase in rate of moulting (percentage columns, Table 4) as was the case with methionine at

TABLE 4
AVERAGE LENGTH AND WIDTH IN MILLIMETERS OF LARVAE TREATED WITH M/250 L-CYSTINE

		Control				Test			
Age	Instar	No.	%	Length	Width	No.	%	Length	Width
48	1	29	73	1.60	.246	46	82	1.61	.255
	2	11	27	2.00	.307	10	18	1.89	.293
72	2	27	90	3.16	.490	30	81	3.13	.473
	3	3	10	3.70	.512	7	19	3.60	.536
96	3	45	100	4.99	.752	36	100	5.11	.761
120	3	44	86	5.04	.798	53	93	5.12	.759
	P	7	14			4	7		

Age in hours from median hour of oviposition
P—pupae

the same concentration. This result is surprising in that, as we shall see later, cysteine is most effective in accelerating the rate of moulting. If metabolism of cystine caused the production of thinner larvae, it might be expected also to increase the rate of moulting as it is converted into cysteine within the cells. On the other hand, it is possible that the thin larvae are only an expression of the specific dynamic effect of cystine without any accompanying modification of other growth processes.

Attempts were made to test the tolerance for high concentrations by dissolving the l-cystine in 0.4 N. hydrochloric acid and testing against a control of equal pH. Under such conditions test and control showed pH 2.2 when M/100 cystine was used. The larvae grew normally and no difference was noted between tests and controls. Higher concentrations increased the hydrogen ion concentration too much for normal growth.

It may therefore be concluded that larvae can tolerate suspensions of l-cystine as high as M/10 without evidence of toxicity or modification of growth processes. Of concentrations between M/10 and M/10,000, only M/250 cystine showed an effect on the larvae. In

TABLE 5

AVERAGE LENGTH AND WIDTH IN MILLIMETERS OF LARVAE TREATED WITH CYSTEINE

		Control				M/50				M/10			
Age	Instar	No.	%	Length	Width	No.	%	Length	Width	No.	%	Length	Width
48	1	76	72	1.64	.26	40	61	1.65	.26	44	100	1.06	.17
	2	29	28	2.23	.33	26	39	2.13	.32				
72	1									14	100	1.23	.18
	2	66	90	3.14	.47	49	77	3.24	.51				
	3	7	10	4.23	.62	15	23	3.82	.58				
96	1									30	75	1.04	.19
	2									10	25	1.78	—
	3	83	100	5.13	.78	28	100	5.33	.82				
120	1									14	45	1.27	.17
	2									17	55	1.71	.25
	3	26	100	5.25	.82	31	89	4.92	.82				
	P					4	11						
		Control				M/250				M/100			
48	1	65	81	1.58	.26	39	71	1.69	.28	55	90	1.65	.27
	2	15	19	2.27	.33	16	29	2.23	.33	6	10	2.54	.39
72	2	61	95	3.04	.47	30	68	3.30	.55	59	81	3.13	.52
	3	3	5	3.75	.59	14	32	4.31	.65	14	19	4.32	.67
96	3	51	100	5.03	.73	45	100	5.46	.81	75	100	5.42	.79
120	3	17	94	5.27	.78	15	94	4.78	.83	15	94	5.06	.83
	P	1	6			1	6			1	6		
		Control				M/5000				M/1000			
48	1	15	20	1.64	.26	11	11	1.58	.26	5	6	1.65	.27
	2	61	80	2.03	.30	88	89	2.07	.31	72	94	2.03	.29
72	2	3	7	3.13	.53	7	7	3.18	.49	4	5	3.19	.46
	3	39	93	4.01	.57	94	93	4.09	.58	89	95	3.96	.55
96	3	19	100	5.21	.83	75	100	5.44	.83	84	100	5.45	.86
120	3	39	95	5.07	.86	7	27	5.10	.93	43	93	5.04	.86
	P	2	5			19	73			3	7		

Age in hours from median hour of oviposition
P—pupae

this case the experimental larvae were at any given length thinner than those in the control medium. Cystine like methionine is concerned in mobilization of fat in vertebrates (Beams, 1946). Perhaps it plays some similar role in *Drosophila* larvae.

Cysteine. Cysteine hydrochloride was added to the basal medium in concentrations between M/10 and M/100,000. The pH of the control was adjusted to the experimental medium by addition of 0.1 N. HCl. All the larvae treated with M/10 cysteine died within 140 hours. A few individuals moulted to the second instar but most of the individuals showed no growth before death. Concentrations of cysteine between M/5000 and M/50 slightly increased the rate of growth in length and width (Table 5 and Figure 2). These concentrations also stimulate faster moulting as shown by the percentage differences for each instar in Table 6. Concentrations below M/5000 were ineffective in modifying any growth processes. No difference in length/width ratios were noted at any concentration.

TABLE 6
PER CENT ACCELERATION OF MOULTING OF LARVAE TREATED WITH CYSTEINE

	1st to 2nd instar	2nd to 3rd instar	3rd instar to pupation
M/50	11	13	11
M/100	5	14	0
M/250	14	3	2
M/500	9	19	3
M/1000	4	25	0
M/5000	8	11	2
M/10,000	—2	3	0

These results are of special interest because of the presence of the SH group in this amino acid. Previous experiments with glutathione and parathiocresol have shown that growth in length of first instar larvae is stimulated by this group (Wilson, 1939). The results reported in the present paper show that not only is the growth in size accelerated, it occurs to such an extent that differentiation proceeds at a faster rate. It is likely that enhanced proliferation and maturation of imaginal cells producing the moulting hormones results in a faster rate of moulting from one instar to the next. Cysteine is much less stable than parathiocresol or glutathione so it would be expected that higher concentrations would be necessary to produce similar results. In addition the presence of partially oxidized derivatives tends to retard growth (Hammett, 1929). The two expressions of

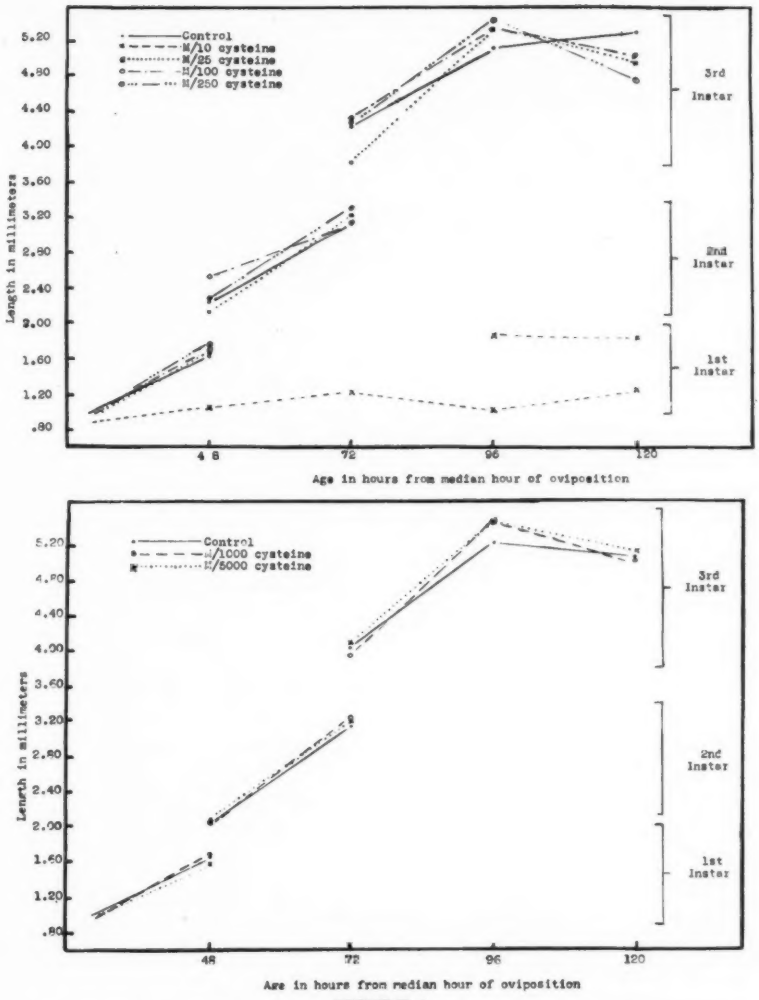

FIGURE 2

RATE OF GROWTH OF DROSOPHILA LARVAE TREATED WITH CYSTEINE HYDROCHLORIDE

growth are balanced at a concentration of M/5000 and lower so that no effect of addition of cysteine is shown by the larvae treated with these lower concentrations.

Of the three sulphur-containing amino acids the results with cysteine showed a reaction to a wider range of concentrations and were much more pronounced. Cysteine is definitely toxic at a concentration of M/10, methionine retards growth and kills some individuals at that concentration while cystine is not toxic at any concentration. Growth is retarded by M/50 methionine but accelerated by M/50 cysteine. Methionine and cystine at a concentration of M/250 accelerated specific processes, while all concentrations between M/50 and M/5000 of cysteine accelerated mass growth and rate of moulting. No differences were noted in the appearance of imagoes for any amino acid at any concentration.

Summary

1. Drosophilae larvae will tolerate dl-methionine in concentrations between M/500 and M/5000, cysteine hydrochloride between M/5000 and M/100,000, and l-cystine in suspensions as high as M/10 without modification of any growth process.

2. Methionine and cysteine are toxic for larvae of Drosophila when added to a basal medium at a concentration of M/10.

3. Methionine in concentrations between M/25 and M/250 retards growth in length and width.

4. Cystine and methionine retard growth in width at M/250 concentration.

5. Methionine accelerates rate of moulting at M/250 concentration.

6. Cysteine stimulates growth in length and rate of moulting at all concentrations between M/50 and M/5000.

References

1. Almquist, H. J. & Grau, C. R. 1945. Further studies on cystine, methionine, and choline in chick diets. *J. Nutrition*, **29**, 219-222.

2. Beams, A. J. 1946. The treatment of cirrhosis of the liver with choline and cystine. *J. A. M. A.*, **130**, 190-193.

3. Beveridge, J. M. R., Lucas, C. C. & O'Grady, M. K. 1945. The effect of dietary proteins and amino acids on liver fat. *J. Biol. Chem.*, **160**, 505-517.

4. Croft, P. B. & Peters, R. A. 1945. Nitrogen loss after thermal burns. Effects of adding protein and methionine to diet of rats. *Lancet*, **248**, 266-272.

5. HAMMETT, F. S. 1929. The chemical stimulus essential for growth by increase in cell number. *Protoplasma,* **7**, 297-320.

6. HAMMETT, F. S. & PADIS, K. 1936. dl-methionine in developmental growth. *Proc. Soc. Exp. Biol. and Med.,* **34**, 181-183.

7. KOTAKE, Y. 1934. Metabolism of proteins and amino acids. *Ann. Rev. Biochem.,* **3**, 193-208.

8. LONG, W. K. & FARAH, A. 1946. Effect of some sulfhydryl-containing substances on the toxicity of organic mercurial compound. *Science,* **104**, 220-221.

9. PORTER, J. R. & MYERS, F. P. 1945. Amino acid interrelationships in the nutrition of *Proteus morganii. Arch. Biochem.,* **8**, 169-175.

10. TOENNIES, G. 1937. The sulphur containing amino acid, methionine. *Growth,* **1**, 337-370.

11. TREADWELL, C. R. 1945. Growth and lipotropism. I. The dietary requirements of methionine, cystine and choline. *J. Bio. Chem.,* **160**, 505-517.

12. WHITE, J. & EDWARDS, J. E. 1942. Effect of dietary cystine on the development of hepatic tumors in rats fed p-dimethylaminoazobenzene (butter yellow). *J. Nat. Cancer Inst.,* **2**, 535-538.

13. WILSON, L. P. 1939. The influence of sulfhydryl and sulfoxide on length growth of first instar larvae in *Drosophila melanogaster. Growth,* **3**, 409-417.

14. ———. 1945. Tolerance of larvae of Drosophila for amino acids: tryptophane. *Growth,* **9**, 145-154.

15. ———. 1945. Tolerance of larvae of Drosophila for amino acids: tyrosine, phenylalanine, and alanine. *Growth,* **9**, 341-352.

16. WILSON, L. P. & BIRCH, M. M. 1944. Tolerance of larvae of Drosophila for amino acids: arginine. *Growth,* **8**, 125-148.

Growth, 1946, **10**, 375-380

THE COMBINED EFFECT OF COLCHICINE AND X-RAYS ON THE GROWTH OF ALLIUM CEPA SEEDS

A. Back

Radiobiological Department of the Cancer Laboratories, Hebrew University, Jerusalem
(Received for publication November 20, 1946)

Dustin and his school were the first to compare the effect of colchicine with that of x-rays on cells. According to their view, the effects of these two agents may have been cumulative. Experiments testing their activity on various types of cells have yielded rather conflicting results. Previous treatment of Protista by L. Halberstaedter and A. Back (2) and of plant cells by M. Levine (5) with colchicine definitely enhanced their reactivity to x-rays. Similar experiments with tumor cells have, on the other hand, generally yielded negative results, although Gayer and Claus (1) reported an enhanced effect on rat tumors when both agents were applied in combination. It would appear that the effect depends on the type of cell tested, the dosage, and on the method of application. In research on colchicine carried out at our Institute, this problem was further investigated, using plant cells as the test object.

Materials and Technics

Our test objects were seeds of Allium cepa (var. Homs), germinated on filter paper at 17° C. Seeds to be tested were exposed to an 0.25% aqueous solution of colchicine (B.D.H.), while controls were exposed to similar quantities of distilled water. The seeds remained in contact with these liquid media until germination had begun, i.e. about 48 hrs. The average root length of seedlings in colchicine and in water at this time was 1 to 1.5 mm. Seedlings as nearly as possible of identical size were rinsed in distilled water and placed on damp filter paper. They were then irradiated or used as unirradiated controls. The following physical constants were used: Siemens Tuto Multivolt Apparatus 160 KV, Ma, 0.5 mm Al. Target distance 23 cm. Intensity 166 r/min. Treated and control seeds were kept at 17° C. for an additional 2 days after which the rootlet was cut off and its length measured.

Results and Discussion

After a series of exploratory tests the x-ray dose of 2000 r was chosen, since no harmful effects on the length or form of the seedlings were noted during the given period of observation after irradiation with this dose, whereas irradiation with 2500 r led to a slight inhibition of growth. Colchicine, alone, in a concentration of 0.25% had no evident influence on the growth of the seedlings, other than the production of thickenings such as have frequently been described among the reactions of roots to colchicine (Havas, 3), (Levan, 4).

It may, therefore, be concluded that neither of these factors, alone, had any demonstrable effect on linear growth in the dosages employed during the given period of observation.

In experiments investigating their combined effects, seeds were divided into four approximately equivalent groups:

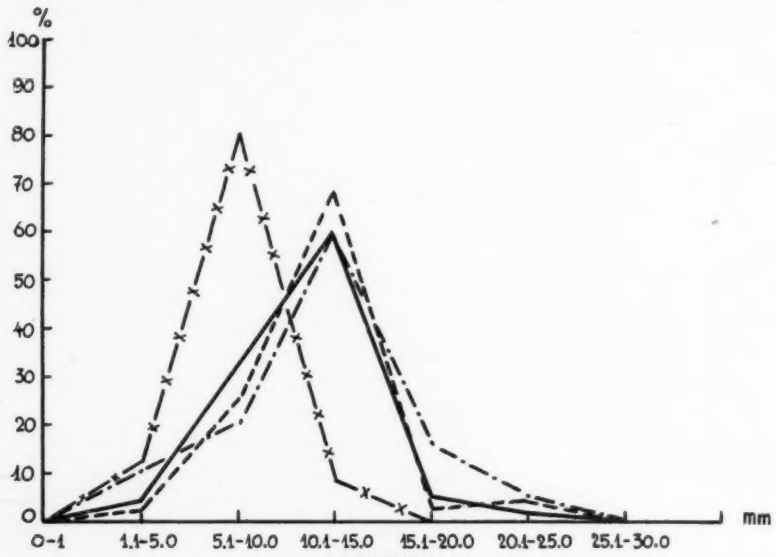

FIGURE 1

Frequency Curves of the Lengths of Rootlets of Allium Cepa Exposed to Varying Conditions

——— Germinated in water.
– – – – – Germinated in water and irradiated with 2000 r.
–·–·–·– Germinated in colchicine (0.25%)
–x–x–x– Germinated in 0.25% colchicine and irradiated with 2000 r.

1) Controls
2) Irradiated seeds not previously treated with colchicine
3) Seeds treated only with colchicine
4) Irradiated seeds previously treated with colchicine.

Rootlets were detached and were ranged in lots of 1 to 5, 5.1 to 10, 10.1 to 15, 15.1 to 20, and 20.1 to 25 mm, respectively. The data thus collected from a total of more than 1500 seeds are presented in Fig. 1. The curves representing seeds treated with either colchicine or x-rays, alone, are similar to the controls. In each of these three groups, 60% to 65% of the rootlets fell within the 10.1 to 15 mm range, while 5% in each group reached maximum lengths of 20.1 to 25 mm. Thus, the peak of the curves lay between 10.1 to 15 mm. On the other hand, the results obtained with irradiated seedlings previously treated with colchicine differed significantly from the controls. In this group 80% of the rootlets fell within the range of 5.1 to 10 mm, while only about 8% reached the maximum recorded range of 10 to 15 mm. Fig. 2 represents photographs of the seven longest seedlings in each of the four groups in one of a series of tests each involving about 100 seedlings. The difference between the combined influence of colchicine and x-rays and each of these factors alone is obvious.

In attempting to analyze the recorded results, it must be assumed that a given dose of colchicine probably does not exert identical effects on all cells, even of the same cell type. Some may be irreparably damaged, so that they are destroyed immediately or at the next cell division. Others may withstand exposure to the toxic effect of the same dose of colchicine, and give rise to normal daughter cells. Between these two extremes may lie still a third group, which does not succumb to the toxic influence of the drug and may undergo subsequent cell divisions, giving rise not to normal cells, but to cells with polyploid or otherwise abnormal nuclei.

Since in our studies linear growth was not demonstrably affected by the given dose of colchicine taken alone, it must be assumed that the number of irreparably damaged cells was not inconsiderable. With respect to the effect of x-rays, our exploratory tests indicated that a dose of 2500 r exerts a definite inhibitory effect on the linear growth of seedlings. It is not improbable that even a dose of 2000 r may give rise to certain changes similar to those induced by colchicine,

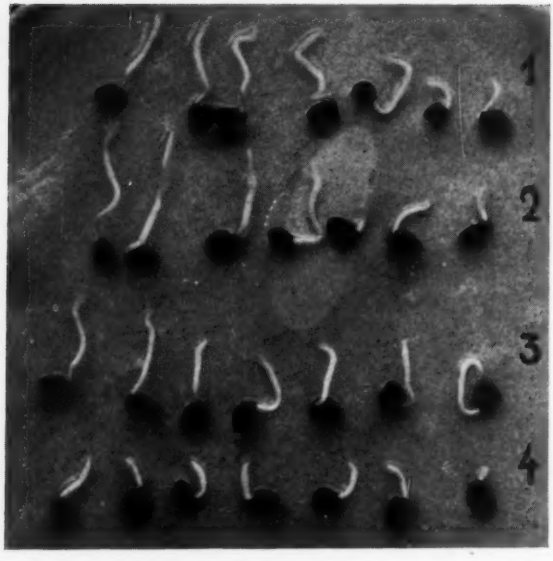

FIGURE 2

PHOTOGRAPHS OF THE SEVEN LONGEST INDIVIDUALS CHOSEN IN A SINGLE EXPERIMENT FROM A GROUP OF 100 SEEDLINGS

1. Germinated in water (Control).
2. Germinated in 0.25% colchicine.
3. Germinated in water and irradiated with 2000 r.
4. Germinated in colchicine and irradiated with 2000 r.

alone, which do not have a measurable effect on the linear growth of the seedlings.

Levine (5), in his experiments on onion bulbs formulated the related view that heightened sensibility to x-ray irradiation after treatment with colchicine is associated with an increase in the number of cells undergoing mitosis, and stated that "the colchicinized were more vulnerable to the x-ray treatment even though the time of exposure occurred when the number of the dividing cells had passed the peak of the reaction to the drug".

How, then, may we explain the augmented inhibitory effect of the two factors, taken together? Cells in no way affected by colchicine must react to x-rays no more intensely than do those not previously treated with colchicine. On the other hand, cells already irreparably

damaged by colchicine require no further inhibition. The chief effect of the combined application must, therefore, be assumed to be an increased sensitivity to x-ray irradiation in those cells undergoing nuclear (polyploid, etc.) changes as a result of previous treatment with colchicine.

TABLE 1

AVERAGE LENGTHS (IN MILLIMETERS) OF ROOTLETS OF ALLIUM CEPA EXPOSED TO TREATMENT WITH COLCHICINE AND X-RAYS IN VARYING COMBINATIONS

No. of Experiments	Controls	Colch.	X-Ray	X-Ray + Colch.
1	11.2	11.3	12.0	8.9
2	14.1	15.2	14.2	9.8
3	13.6	13.9	14.2	8.2
4	14.6	—	13.2	7.1
5	7.1	—	6.2	3.9
6	9.4	—	9.6	7.5
7	8.8	—	9.0	—
8	8.1	—	8.1	—
9	9.8	—	9.9	—
10	9.6	—	9.8	—
11	11.5	—	11.1	—
12	—	7.2	—	3.7
13	—	8.5	—	3.6
14	—	9.7	9.4	6.3
15	—	12.0	11.9	9.1
16	—	—	8.1	5.5
17	—	—	13.1	7.6
18	—	—	13.6	7.7

SUMMARY

It has been demonstrated that colchicine in a 0.25% aqueous solution or a dose of x-rays (2000 r), separately applied, have no measurable effect on the length of seedlings of Allium cepa.

The growth of seedlings of Allium cepa treated with 0.25% colchicine solution before the application of an x-ray dose of 2000 r is inhibited by about 20%.

This inhibition may be explained if it be assumed that colchicine, by including nuclear changes (polyploid, etc.) sensitized a considerable number of cells to x-ray irradiation.

These results confirm former observations on paramaecium and onion bulbs.

References

1. Gayer, M. & Claus, P. 1939. Irradiation of cancer following injection of colchicine. *Proc. Soc. Exper. Biol. & Med.*, **42**, 565.
2. Halberstaedter, L. & Back, A. 1943. Influence of colchicine alone and combined with x-rays on Paramaecium. *Nature*, **152**, 275.
3. Havas, L. 1937. Effects of colchicine on Allium and of Visc. album preparations upon germination of seeds and growth of seedlings. *Nature*, **139**, 371.
4. Levan, A. 1939. The effect of colchicine on Meiosis Allium. *Hereditas*, **25**, 9.
5. Levine, M. 1945. The effect of colchicine and x-rays on onion root tips. *Cancer Research*, **5**, 107.

Growth, 1946, **10**, 381-390

NITRATE AND PHOSPHATE vs. L-PROLINE AS FACTORS IN THE BEHAVIOUR OF *Ulva*[1]

PHILIP R. WHITE

WITH THE TECHNICAL ASSISTANCE OF ROBERT S. MELVILLE, ELEANOR V. MELVILLE, AND ELSA HUTZLER

(Received for publication December 2, 1946)

In 1943 Walp (11) made studies in which disks cut from vegetative sheets of *Ulva lactuca* were treated with various concentrations of l-proline added to filtered sea water. From the results he concluded that l-proline at a concentration of 0.05 mM brought about an increase in disk diameter, and an increase in chlorophyll content as measured colorimetrically. He concluded that l-proline "does act to forward differentiation in plant tissue as it does in animal". Hammett and Walp drew a similar conclusion from studies on the effect of l-proline on the development of sporelings of *Fucus spiralis* (3).

These results were obtained by adding l-proline to filtered sea water using sea water without l-proline as a control. No other controls were used. Two questions therefore arise: (1) were the effects observed due to l-proline as such or might they have been duplicated by some other amino-acid, and (2) if not specific for l-proline, were they specific for amino-acid or might they have been duplicated or approximated by some other source of nitrogen? It has long been known that in sea water in the temperate zones, at the surface and in the summer, the concentrations of both nitrogen and phosphorous fluctuate widely as a result of their utilization by plant life (6, 10, 12). In the neighborhood of Plymouth, England, for example, the phosphate concentration drops in August almost to the disappearing point (1). These two ions become limiting factors in the growth of plant life (1, 2, 4, 5, 8). They might be such factors in the metabolism of *Ulva* and *Fucus*, and there is nothing in the procedures used in Walp's and Hammett's experiments to preclude the possibility that the observed effects of l-proline might have been due to its nitrogen

[1]Aided by a grant from the Women's Auxiliary of the Lankenau Hospital Research Institute.

content rather than to either its amino group or its pyrrolidine ring. If the effect was due to the nitrogen content, as such, then the possibility of another amino-acid having a like effect becomes without significance. It was therefore decided to examine this question in as simple a fashion as possible, using procedures comparable with those employed by Walp.

Materials and Methods

Sea water was collected in carboys on the open ocean side of the Cape, at Highland Light. As soon as it could be brought to the laboratory it was filtered by suction through double layers of ash-free quantitative filter paper on a Buchner funnel, into clean carboys. The requisite chemicals were added to this filtrate. Solutions were fed into finger-bowls by a system of constant level siphons and drained off through capillary drip tubes so arranged that solution was supplied at intervals with a rush which thoroughly mixed the contents, simulating the flow of the tides. The siphons were adjusted to change the water about once every three hours. *Ulva lactuca* was collected at low tide at the Provincetown breakwater. Fronds were chosen where they were in deep, free-flowing water and were selected for uniformity, color, thickness, freedom from epiphytes, and freedom from sporulating areas. Preference was given to large fronds. They were placed in fresh sea-water in clean enameled pails and transported immediately to the laboratory. Here a large frond was spread out in a tray whose bottom had been coated with a 3 mm. sheet of paraffin and was covered with about a centimeter of sea-water. Disks were cut with a sharp 10 mm. cork borer, choosing as far as possible uniform areas free from flaws. In general a single frond was used for an experiment so as to preclude individual differences entering into the results. Differences due to position in the frond (tissue age) could not be eliminated by this means, but by cutting about 300 disks at a time before transferring to the finger bowls, some random distribution was obtained. Thirty disks were placed in each finger bowl. Experiments were continued for one week. At the end of three days, eight disks were selected as being average examples of the contents of each dish, were mounted on a slide and examined, records being made of extent of sporulation, distribution of chromatophores, condition of pyrenoids, etc. At the end of one week, ten disks were removed from each dish for colorimetric examination. Eight of the

remaining fourteen disks were again mounted and carefully examined, complete records being kept. Their diameters were measured to within a half millimeter and the measurements averaged. A single example chosen as typical of each result was photographed.

Three substances were compared—l-proline, $NaNO_3$, and Na_2HPO_4. In the case of proline the concentration found by Walp to be optimal (0.05 mM) was taken as a standard and five solutions prepared, representing 0.1, 0.3, 1.0, 3.0, and 10.0 times this concentration (0.005, 0.015, 0.05, 0.15, and 0.5 mM). With a single atom of nitrogen per molecule this gave the same *nitrogen* concentrations as equimolar amounts of $NaNO_3$. A standard concentration of $NaNO_3$ was chosen to approximate the winter nitrate level of surface sea water at the latitude of Cape Cod (0.1 mM) (4, 5), and a similar series of concentrations was prepared (0.01, 0.03, 0.1, 0.3, and 1.0 mM). These cover the same range of nitrogen concentration as those chosen for proline. Phosphate concentration was likewise chosen to approximate the winter surface phospate of sea water of the latitude of Cape Cod (0.0003 mM) (1, 2, 8). The series was 0.00003, 0.0001, 0.0003, 0.001, and 0.003 mM. The cultures were placed in diffuse light. To obviate possible effects of differences in illumination each experiment was repeated, using a different arrangement of dishes with respect to windows at each repetition. The variations in illumination appeared, however, to have no significant effect.

Results

The results of a typical experiment, run from August 13 to August 20, 1946, will be presented. For the sake of brevity the results will be described in detail for only a few solutions.

1. *Control,* filtered sea water. "Very little marginal breakdown, circles with uniform margins, pale, no sporulation. Chromatophores all retracted to one side of the cell as by plasmolysis. Condition definitely poor." (Fig. 1,a).

2. *Proline,* 0.005 mM (= one tenth of Walp's optimum. Among proline solutions this gave the best result in our experiments). "No marginal breakdown, circles with uniform margins, blue green, no sporulation. Chromatophores generally well expanded though occasionally plasmolyzed. Condition poor." (Fig. 1,b).

3. *Nitrate,* 0.03 mM (This gave the best qualitative result of any nitrate concentration tested. It has six times the nitrogen content of

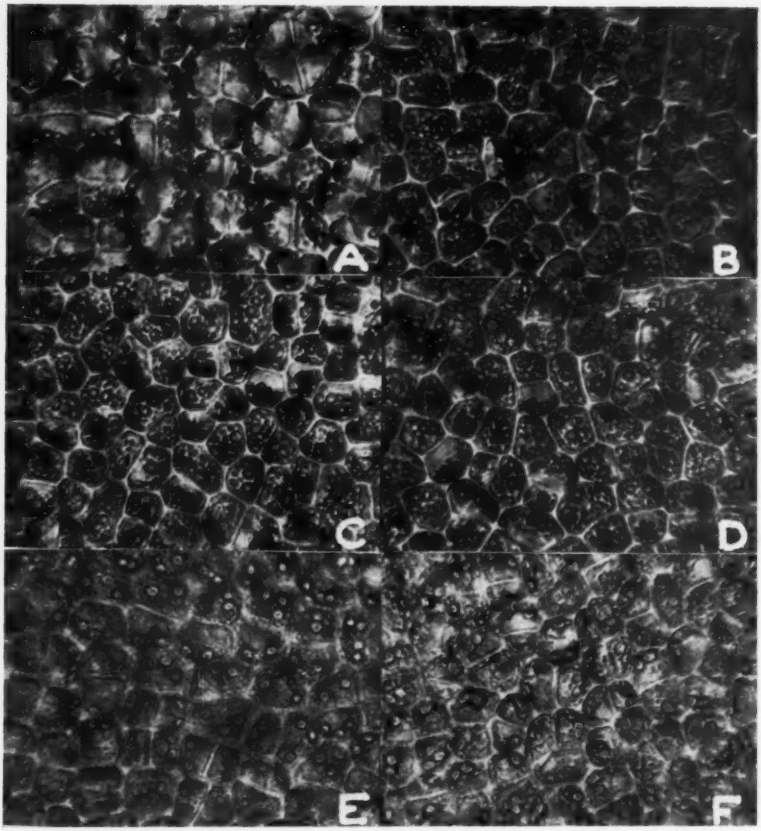

FIGURE 1

PHOTOGRAPHS OF COMPARABLE AREAS OF DISKS OF *Ulva lactuca* GROWN FOR 7 DAYS IN DIFFERENT SOLUTIONS

(a - e were originally cut from a single frond and were grown simultaneously; f was from a later experiment. All photographs x 300.)

a. control. Sea water, filtered but otherwise unmodified. The chromatophores are retracted against one side of each cell, leaving a clear space. The two (or three) round pyrenoids are somewhat obscured by the shrunken chloroplasts and by the close packed but irregular and eroded starch grains. The cells appear larger than those in b - e, but this is an optical illusion due to the chloroplast retraction taking place in such a manner as to leave the open spaces of three or four adjacent cells together, giving the impression of a single large vacuole.

b. l-proline, 0.005 mM. Chromatophores mostly expanded, in some cases retracted. Pyrenoids evident, starch grains numerous, regular, not eroded.

c. nitrate, 0.03 mM. Chromatophores expanded. Pyrenoids evident, starch grains numerous, regular, not eroded.

d. phosphate, 0.001 mM. Chromatophores expanded. Pyrenoids evident, starch grains numerous, regular, not eroded. Compare b, c, and d, which are indistinguishable so far as those characteristics depicted in these photographs are concerned.

e. nitrate, 1.0 mM. Chromatophores expanded. Pyrenoids evident, starch grains entirely wanting, replaced by a finely granular structure.

f. nitrate, 1.0 mM; phosphate, 0.001 mM. This is to be compared with d and e. The chromatophores are expanded. The pyrenoids are larger than when either nitrate or phosphate was supplied alone. As in e there is no evident starch but the general chromatophore background is much more coarsely granular than in e.

the best proline solution). "Some marginal breakdown, dark green, considerable areas of sporulation. Chromatophores mostly well expanded. Condition excellent." (Fig. 1,d).

4. *Nitrate,* 1.0 mM. "Margins badly eroded. Some sporulation. Chromatophores fully expanded. Condition fine." (Fig. 1,e.).

5. *Phosphate,* 0.0001 mM (This gave the best results of any phosphate concentration tested). "No marginal breakdown, no sporulation. Uniformly dark yellow green. Chromatophores fully expanded. Condition fine." (Fig. 1,c).

The results, as recorded in the photographs and in the subjective descriptions of microscopic findings were, it is believed, significant and important. In the control solution, sea water, the chromatophores (7, 9) were regularly retracted against one side of the cell and the granules contained therein (starch) were irregular in outline (Fig. 1,a). Because of the high amount of light transmitted the disks appeared pale in color as compared with the experimental sets. The general impressions both upon gross and microscopic examination was poor. Nitrate, phosphate, and proline, all three, produced marked improvement. The microscopic picture (Fig. 1, b, c, d) at optimum concentration was indistinguishable among these three—the chromatophores were expanded to cover the entire bottom (surface away from the light source) and sides of the cells and were filled with large, regular granules (starch). Only the gross picture showed pronounced differences. All three sets of disks in the experimental solutions appeared darker, richer green than the control.

Numerical results on final diameter of disks, color intensity per disk, and average area of cells are given in table 1. In only a single case—0.3 mM NO_3—did the average disk diameter exceed that in the control by as much as 10%. This by itself would be of doubtful significance, but since all cultures in nitrate solutions showed some increase in diameter as compared with the control (table 1) this can be taken to suggest a growth stimulating effect. Moreover there was also a slight increase in average cell area (113%) in the high concentration of nitrate (1.0 mM; fig. 1,e; table 1), evidence tending to the same conclusion. The colorimeter readings for nitrate (table 1) showed a somewhat deeper color than the control. This, however, gives a somewhat inadequate picture. With increased nitrate concentration there was an increasingly marked marginal erosion. This was due to destruction of marginal cells by bacteria and protozoa

TABLE 1

EFFECTS OF TREATMENT OF *Ulva lactuca* WITH L-PROLINE, $NaNO_3$, AND Na_2HPO_4

		Control	l-proline					$NaNO_3$					Na_2HPO_4					
mM			mM					mM					mM					1.0 mM NO_3, 0.001 mM PO_4
			0.5	0.15	0.05	0.015	0.005	1.0	0.3	0.1	0.03	0.01	0.003	0.001	0.0003	0.0001	0.00003	
Average diam. of disks[1]	Final results	100	101	101	103	102	102	103	110	103	104	105	99	98	98	101	100	—
Average area of cells[2]		100	—	—	—	—	98	113	—	—	100	—	—	—	—	101	—	83
Colorimeter, T/C[3]		100	105	124	130	121	115	135	99	102	93	116	99	101	89	90	119	—
Figure reference		a	—	—	—	—	b	e	—	—	d	—	—	—	—	—	c	f

[1]Average of 10 disks; maximum and minimum diameters measured to nearest 0.5 mm and averaged.

[2]Area of 10 cells measured on photographs (figure 1) with a planimeter and values averaged.

[3]Ten disks placed overnight in 2 ml. distilled H_2O, then heated to 100° C. in oven for 30 minutes to kill cells. Water decanted and 4 ml. 95% ethyl alcohol added, shaken, and placed in the dark for 15 minutes. The alcoholic extract then decanted and color intensities of the extract read in Dubosq colorimeter. The figures are the averages of eight readings, four by each of two observers.

which grew profusely in nitrate cultures. Apparently sea water, being deficient in nitrate, does not support growth of these epiphytes. Where bacterial erosion occurs, the surviving areas are always dark green with well expanded chromatophores and obviously in good condition. But this bacterial erosion results in a reduction of green *area*. Since the unit of comparison in colorimetric measurements is the original disk, these eroded disks would naturally give lower readings than would uneroded disks of equal color intensity. When, therefore, disks grown in nitrate (eroded) give readings equal to or only slightly darker than the controls (uneroded) we must weight this result by an "erosion factor", and say that disks grown in a nitrate-enriched solution contained much more chlorophyll than either the controls or disks grown in a phosphate-enriched solution. When, on the other hand, disks grown in l-proline (uneroded) showed a greater colorimetric reading than most of the others (table 1) we may say that this indicates a greater chlorophyll content than the controls or phosphate disks but it must be discounted (by this same erosion factor) when comparing them with those grown in nitrate. Cultures grown in l-proline lacked the yellow tone of the nitrate and phosphate cultures, being bluish green.[2] Those grown in phosphate had an especially brilliant yellow-green color. Nitrate at optimal concentration produced somewhat more sporulation than did phosphate, l-proline or control.

In high (toxic) concentrations of l-proline and phosphate the chromatophores tended to retract as in the control, leaving vacuolar spaces. Nitrate, on the other hand, produced no clearly toxic response at any concentration tested. At 1.0 mM nitrate (a level at which bacterial and protozoan erosion was too great to permit long survival) the chromatophores were as fully expanded as at the optimum concentration (0.03 mM). At this level, however, the larger granules were almost completely lacking so that the two or sometimes three pyrenoids stood out sharply against a finely granular background (fig. 1,e). Whether this indicates a shift from storage of starch to that of sugar was not determined microchemically. At this level the cells are somewhat larger (table 1) than in any other solution (113%).

Figure 1,f is included as suggestive of certain other possibilities.

[2]The necessity of comparing yellow greens and blue greens in the colorimeter of course renders doubtful the significance of these quantitative figures.

In this culture a concentration of 1.0 mM nitrate and 0.001 mM phosphate were applied simultaneously. The general picture is somewhat similar to that at 1.0 mM nitrate alone, but the pyrenoids were somewhat larger and the background somewhat more coarsely granular. The cell areas were *definitely* smaller (17%). This may be merely the result of origin from a different frond, for this example was taken from an experiment carried out two weeks later than those in figures 1, a-e. There is, however, another possibility. Examination of the picture shows a very large percentage of cell pairs (11 pairs among 87 cells), indicating that cell division was either presently active or recently so. Small size is of course commonly a concomitant of rapid division. In fig. 1,b there are only two evident pairs, in 1,c none, and in 1,e possibly two. This may indicate, therefore, that when both phosphate and nitrate are present the tissue becomes actively meristematic, as it cannot do in the absence of either ion. A culture period of more than seven days might have resulted in significant growth under these conditions.

Discussion

From the results presented above, certain facts emerge clearly. Sea water, as collected on Cape Cod in July and August, does not provide optimal conditions for growth of *Ulva lactuca*. It can be improved by the addition of either nitrate or phosphate. The amount of phosphate required to bring about marked improvement is extremely low (10^{-7} M) so that it is clear that sea water is deficient in this ion almost to the vanishing point. While addition of l-proline to sea water will likewise bring about an improvement in metabolism of *Ulva,* this improvement is not so great as when nitrate is added. It is clear that nutritional studies can be interpreted with assurance only if a sea water adequately fortified with these two ions is used as a base. Sea water is not a satisfactory control when studies are to be made on the nutritional effects of substances containing either nitrogen (as does l-proline) or phosphorous. Walp's conclusion, that l-proline specifically promotes differentiation in *Ulva,* and Hammett and Walp's conclusion that l-proline promotes differentiation in *Fucus* appear, therefore, to be without sufficient foundation. Unless such a specific promoting effect can be demonstrated when l-proline is superimposed on an optimal nitrate level it cannot be considered to have been unequivocally established.

That *both* nitrate and phosphate should promote development when supplied separately appears somewhat surprising, for if low phosphate level is limiting no amount of nitrate alone should correct the deficiency and *vice versa*. This result appears explicable only if the addition of each serves to free and make available bound residues of the other. These, however, are questions which must be answered by future work.

Moreover, it is clear, from the bacterial erosion which occurs when adequate nitrate supplies are present, and the derangement which this erosion introduces into quantitative measurements such as diameter of disks, colorimetry, etc. that really satisfactory, clearly interpretable results can only be obtained under aseptic conditions. This imposes a very important burden on the experimenter. Surface sterilization of experimental material is not likely to succeed without introducing unknown physiological variables. It is probable that spores can be sterilized and aseptic sporelings raised and this will probably have to be done. This raises an entirely new set of problems which will have to be solved before this type of investigation can be carried much further.

It is perhaps significant to note that *Ulva lactuca* grows profusely in the pools adjacent to the Provincetown breakwater, occurs sparingly on the bay shore, and is completely wanting along the open ocean shore. Proximity to Provincetown probably results in a local elevation of both nitrate and phosphate content of the water, from decomposing offal of various sorts which are lacking in the open ocean. This may very possibly be a significant factor in the ecology of *Ulva*.

References

1. Atkins, W. R. G. 1923. The phosphate content of fresh and salt water in its relationship to the growth of the algal plankton. *J. Marine Biol. Assoc.*, **13**, 119-150.
2. ————. 1926. A quantitative consideration of some factors concerned in plant growth in water. *J. Conseil Perm. Internat. Explor. Mer*, **1**, 99-126, 197-226.
3. Hammett, F. S. & Walp, L. 1943. The differentiation-forwarding effect of l-proline on eggs of *Fucus*. *Growth*, **7**, 199-217.
4. Harvey, H. W. 1926. Nitrate in the sea. *J. Marine Biol. Assoc.*, **14**, 71-88.
5. ————. 1928. Nitrate in the sea. *J. Marine Biol. Assoc.*, **15**, 183-190.
6. ————. 1928. Biological Chemistry and Physics of Sea Water. Cambridge Univ. Press. London. 194 pp.
7. Oltmanns, F. 1905. Morphologie und Biologie der Algen. II Bd. Fischer, Jena. 443 pp. (p. 95, 113).

8. REDFIELD, A. C. 1934. On the proportions of organic derivatives in sea water and their relation to the composition of plankton. James Johnstone Mem. Vol. Liverpool Univ. Press. 176-192.

9. SCHMITZ, F. 1882. Die Chromatophoren der Algen. Bonn.

10. THOMPSON, T. G. & ROBINSON, R. J. 1932. Chemistry of the sea. (Physics of the Earth. V.) *Oceanography*. Bull. Nat. Res. Council, **85**, 95-203.

11. WALP, L. 1943. Effect of l-proline on differentiation in Ulva. *Growth*, **7**, 37-45.

12. ZOBELL, C. E. 1946. Marine Microbiology. *Chronica Botanica*. Waltham, Mass. pp. 240.

Growth, 1946, **10**, 391-410

THE MECHANISM OF FERTILIZATION OF EGGS*

MATILDA MOLDENHAUER BROOKS

The Marine Biological Laboratory, Woods Hole, Mass., and The University of California, Berkeley, Cal.

(Received for publication December 10, 1946)

These experiments form the basis of a new explanation for some heretofore unexplained phenomena concerning the process of fertilization of eggs. What factors does the sperm add to the egg that starts development? Why does the egg develop under certain conditions without sperm? These questions have been the source of many experiments. The hypothesis of the writer (Brooks, 1943) that fertilization depends upon the establishment of an appropriate redox potential of the oxidative enzymes of the egg is extended in this paper.

The problem of development of the marine eggs used in these experiments, consist of two steps: formation of the fertilization membrane, and cleavage. The many experiments of Loeb, 1913, Lillie, 1911, Heilbrunn, 1915, Hollingsworth, 1941, Chase, 1935, Moser, 1940 and others demonstrate that these two steps are not necessarily related, and coincidental. They can be produced separately by artificial means, and cleavage can occur without membrane formation. According to Lillie, 1911, oxygen is not always necessary for membrane formation but it is for cleavage. In some cases dilute concentrations of KCN can be used to produce membranes, as in *Asterias*, but KCN must be removed before cleavage is begun.

In a preliminary paper (Brooks, 1943), the writer has demonstrated certain important relations between O_2 consumption and the aerobic oxidation-reduction potential of three different species of eggs, and has suggested that the redox potential is the important factor in determining the various reactions of different eggs to substances causing activation. It has been shown by Genevois, 1928, Barron, 1930 and Baumberger, 1939, that as the redox potential of cells becomes more positive within certain limits, the rate of O_2 consumption is

*Preliminary Report, Federation Proceedings, March 11, 1946. The expenses of this investigation were in part defrayed by the Fellowship Fund of Kappa Alpha Theta.

increased and vice versa. The term, redox potential, as applied in this paper, refers to the electron pressure measured in millivots, on a gold-plated platinum electrode immersed in a thick suspension of living cells. A Coleman electrometer was used. In addition, the H ion concentration of this suspension was determined by a glass electrode. These two sets of readings, E_h and p_H respectively, are combined in the term, r_H, or redox potential, which is a function of these two factors as follows: $r_H = 2\ p_H + \frac{E_h}{.03}$. This term is taken from Clark, 1928, and assumes that the slope of the E_h-p_H curve is .06 within the range of p_H used here.

The redox potential represents a function of the ratio of the sum of the reductants to the sum of the oxidants of the electromotively active and reversible systems of the oxidative enzymes and other reactants. These enzymes govern respiration, and most of the energy of the cell is derived by their reactions. When the ratio deviates from unity, other factors being equal, the rate of O_2 consumption declines. This appears to be the case in unfertilized *Arbacia* eggs where the rate of O_2 consumption is low. We can interpret this as an example of the large preponderance of reductants over oxidants. On the other hand, in unfertilized *Asterias* eggs and in *Chaetopterus*, the rate is high. This means that here the rate of oxidants to reductants is more nearly equal. There is a correlation between the redox potential and the O_2 consumption, and this theory is herewith tested, by taking measurements of three different species of both unfertilized and fertilized eggs, sperm and larvae, and of different artificial solutions causing fertilization. The question then is, what is the redox potential of sperm and of artificial solutions causing artificial parthenogenesis, as compared with that of the egg?

This theory was examined in three different cases: where there is a rise in the rate of O_2 consumption on fertilization, as in *Arbacia punctulata;* where there is only a slight rise, as in *Asterias* forbesii; and where there is a decrease, as in *Chaetopterus pergamentacea.*

Methods

The eggs of the three marine species were strained through a fine mesh cloth into filtered sea water. At the time of taking the readings, they were centrifuged at 2000 RPM at a radius of 13 cm. for ½ minute, using 15 cc. centrifuge tubes or pointed 50 cc. tubes.

From these packed eggs, one cc. was placed in a small Pyrex glass vessel where readings were made immediately for E_h and p_H. Sperm was measured "dry", directly as it was taken from the animal without being centrifuged, care being taken not to contaminate it with echinochrome or sea water. Sperm was not placed in sea water as in the case of the eggs, because it is not possible to centrifuge it from the sea water except at a very high rate of speed, so that equal treatment of sperm and egg could not be accomplished. In any event, the small residual concentration of sea water remaining around the eggs after centrifugation and decantation, would tend to raise the redox potential of the eggs to a higher level than that which would be obtained in the complete absence of sea water. In other words, any error in the measurement of the eggs would tend to increase the potential rather than to lower it.

In taking the readings, about one minute was necessary for establishing equilibrium between the electrode and the cells. This time, in which two to three successive readings were the same, was used as the equilibrium. After this a drift in potential occurred. All readings were taken with freshly cleaned electrodes. Four such electrodes were in use and served as a check against each other. For further information on handling the electrodes, see Clark, 1928, page 328. All measurements were made under similar conditions of time and manipulation, so that errors in the method would be constant. The temperature varied during the season at Woods Hole from 20° C. at the beginning to 25° C. during the summer months. The temperature remained sufficiently constant during any one day to avoid errors in this regard.

The stages of eggs measured were unfertilized, fertilized, 2- to 4-cell, 8-, 16-, 32-cells, morula, non-swimming and swimming blastula, gastrula, pluteus at different stages or bipinnaria in *Asterias,* the same stages in *Chaetopterus,* and sperm of each. Since the eggs did not always develop at the same rate, it was not possible to measure only one stage, but in each case, the measurements were taken when the majority were in the stage indicated. Fertilized eggs were rinsed several times with sea water to clear them of sperm after the membranes had been formed. Larvae were spread out in a thin layer in large crystallizing dishes for development, and the water changed daily. They were prepared for experimentation as needed. The time required for the development of the different stages varied somewhat as the season progressed. The summary is indicated in the tables.

Comments on Relations Between r_H of Interior and Exterior of the Cell

In these experiments, measurements were made of the packed cells. Between the cells a small amount of sea water remains after centrifuging. Measurements are therefore of the milieu, and the redox potentials obtained are the resultants of the factors produced by the organisms, such as CO_2 production, acid production, and other products of metabolism. It might be objected that the internal r_H value is not the same as the external. The only experiments in this connection are those of Chambers, Pollack and Cohen, 1929, who microinjected redox dyes into eggs of *Asterias forbesii*, and found the r_H to be around 12. When the same species of eggs was immersed in solutions of redox dyes, a similar r_H value was obtained for the external milieu (Chambers, Beck and Green, 1933). These experiments show that there is a direct relation between the redox potential of the outside of the egg and the inside. Rapkine and Wurmser, 1926, microinjected the European species of sea urchin and starfish eggs and found an r_H of 19 to 20. These values are higher than those obtained by the writer, and differences are probably due to experimental methods. The fact that reagents can produce artificial parthenogenesis in the egg, is evidence that there is a direct relation between the redox potential of the inside and that of the outside of the egg. Further proof of the relation between exterior and interior of the egg is shown by the experiments with KCN. This reagent lowers the redox potential, depending upon its concentration, according to Barnard, 1933. If the redox potential of the organism being tested is already negative, KCN should have little effect, depending again upon the concentration. On the other hand, if the potential is high, it should be affected by KCN. These two conditions are illustrated by unfertilized *Arbacia* eggs which have a low redox potential and are not affected by KCN, and by fertilized *Arbacia* eggs which are affected by KCN. In the latter case, all evidence points to the conclusion that fertilization has raised the potential of the egg, but only temporarily. And finally, that there is a definite relation between the reducing intensity of cell suspensions and of physiological systems in general and their physiological condition is found in the later work as stated by Bull, 1943.

The redox potentials of certain hypertonic solutions without eggs

in some cases and with eggs in other cases, as used by Loeb, 1913, and others for producing artificial parthenogenesis in certain marine eggs was also measured as shown in Tables 4 and 5. These were hypertonic solutions of NaCl, $CaCl_2$ and $MgCl_2$ in sea water, butyric acid solutions in sea water and KCN in hypertonic sea water; in addition, the effect of eggs in producing changes in the redox potential of some of these solutions including hypotonic sea water and artificial sea water was measured as seen in Table 5.

When eggs such as *Arbacia* which have a low redox potential, are placed in a solution with sperm which has a high potential, it is assumed that there is an interchange of electrons according to the laws of oxidation-reduction and those of reaction kinetics. This electron shift results in a change in the ratio of oxidants to reductants of the enzyme systems in egg and sperm, resulting in the so-called "activation" of the egg. The redox potential of the egg is adjusted by the inter-exchanging electrons from these systems of enzymes. It is known that about 70% of the energy of the cell is produced by these oxidative enzymes. Through the release of this energy, the processes of development are set in motion, and the unfertilized egg is "activated".

Reference to Table 5 shows that eggs give off substances that lower the potential. This is seen in experiments in which large numbers of eggs are placed in a small volume of sea water. It is particularly demonstrated in the case of centrifuged masses of eggs in a minimum volume of sea water. On the other hand, if one egg were suspended in a large volume of sea water, the effect of the substances given off on the redox potential of the milieu would be practically nullified. It is undoubtedly for this reason that developing eggs must be placed in a sufficient volume of sea water to promote their development.

O_2 Tension: Electrodes in the Presence of Oxygen

These experiments were performed aerobically because it was thought that anaerobic conditions would not give the true picture of organisms which live in air. Since uniform suspensions of organisms were used and uniform times for taking the measurements, it is assumed that any errors resulting from methods would be constant. The small volumes of eggs or sperm used afforded access to adequate diffusion of air so that a partial anaerobic state could be ruled out.

Commenting on these aspects of experimentation in connection with electrode measurements of cell suspensions under aerobic conditions, Clark, 1928, states, "electrodes in the presence of atmospheric oxygen are able to induce definite potentials. Indeed there is no need to exclude air in crude measurements of some reversible systems—a fact which indicates the comparatively inert nature of molecular O_2." Furthermore, in the same connection, a "suspension of washed cells under anaerobic conditions is unable to reduce methylene blue but does reduce the indophenol dyes in the presence of a vigorous stream of air passed through the suspension", is proof that what is being measured is the actual oxidation-reduction intensity of the enzymes rather than changes in the oxygen tension of the solution. Clifton et al. (1934), Rapkine (1930) and others have shown that anaerobic organisms can grow in the presence of air provided that dyes of a low redox potential are added to poise the solution.

Amberson, 1928, found that O_2 consumption is independent of the O_2 tension over a wide range for many organisms. In the case of *Arbacia* eggs he found that the O_2 consumption continues at a normal rate when the pressure of O_2 varies from 228 mm. Hg to 20 mm. Hg, thus corroborating the earlier experiments of Warburg, 1908, who made similar observations on the European sea urchin egg.

This apparent lack of effect of O_2 tension can be explained by Clark, 1935. He states that the full release of the inherent ability of oxygen to become activated through enzymes does not occur, for then there would be no withstanding the destructive action of the oxygen. However, if oxygen can enter into the reaction only as it converts the catalyst systems to the oxidized components, it is restrained.

Since the rate of oxygen consumption is a direct function of the electrode potential according to Genevois, 1928 and Barron, 1930, one can infer that there would be no change in the electrode potential within the range of possibly reduced oxygen pressure such as the case cited above. The present set of experiments would also not be affected by any possible slight change in O_2 tension due to crowding under the conditions used there.

We can therefore conclude that what is being measured is not a change in the O_2 tension of the solution or the milieu, but the state of the electromotive activity of the oxidation-reduction enzymes.

Results

It has been previously shown by the writer (Brooks, 1943) that a change in the redox potential of *Arbacia* eggs occurs as they develop. In these experiments, details of the experiments on *Arbacia* eggs are given along with those on *Asterias* and *Chaeptopterus*.

The three types of eggs gave the following results:

1. In *Arbacia*, as shown in Table 1, the unfertilized egg has a low

TABLE 1

Summary of Results with *Arbacia*
Experiments with *Arbacia*

Column 1, the number of experiments at each stage (Column 2); Column 3, the E_h or electrode potential in millivolts; Column 4, p_H; Column 5, the time after fertilization; Column 6, r_H or redox potential.

1	2	3	4	5	6
No. of experiments	Stage of development	E_h in millivolts	P_H	Time after fertilization	$r_H = 2\ p_H + \frac{E_h}{.03}$
30	Unfertilized eggs	—.269	6.58		4.20
30	Sperm	—.188	7.41		8.62
32	Fertilized eggs	—.280	6.70	20 minutes	4.10
23	2-cell division	—.278	6.43	80 "	3.70
15	4-cell division	—.283	6.45	123 "	3.80
12	8-cell division	—.288	6.59	162 "	2.58
15	16-cell division	—.283	6.45	203 "	3.50
14	32-cell division	—.277	6.74	266 "	4.28
10	Blastula, non-swimming	—.268	6.55	464 "	4.20
16	Blastula, swimming	—.265	6.66	687 "	4.52
19	Gastrula	—.267	6.94	1214 "	4.98
40	Pluteus, early	—.289	7.15	2 days	5.70
25	Pluteus, later	—.287	7.02	3 days	5.84
18	Pluteus, later	—.280	7.22	4 days	5.10
5	Pluteus, later	—.279	7.20	5 "	5.10
6	Pluteus, later	—.277	7.10	6 "	5.0
3	Pluteus, later	—.281	7.10	11 "	4.9

redox potential while the sperm has a much more positive one. This corresponds with the results on the rate of O_2 consumption which is low in the former case and high after fertilization as shown by Warburg, 1910, and many others after that time. During division, the redox potential at first drops but gradually rises again until the pluteus stage is reached. When plutei are kept without being fed, they gradually become smaller and the redox potential drops.

2. In *Asterias*, as demonstrated in Table 2, the unfertilized egg has a high redox potential. Sperm is only slightly higher than that

TABLE 2

SUMMARY OF RESULTS WITH *Asterias forbesii*
EXPERIMENTS WITH *Asterias Forbesii*

Column 1, the number of experiments at each stage of development (Column 2); Column 3, the E_h or electrode potential in millivolts; Column 4, p_H; Column 5, time after fertilization; Column 6, r_H or redox potential.

1	2	3	4	5	6
No. of experiments	Stage of development	E_h in millivolts	P_H	Time after fertilization	$r_H = 2\ p_H + \frac{E_h}{.03}$
12	Unfertilized eggs	—.180	6.99		7.99
5	Sperm	—.197	7.30		8.00
12	Fertilized eggs	—.205	7.15	29 minutes	7.50
8	2-4-8 cells	—.152	7.56	249 "	8.92
12	8-16-32 cells	—.179	7.53	6 hours	9.26
3	Blastula, non-swimming	—.164	6.95	19 "	8.50
8	Blastula, swimming	—.190	7.18	22½ "	8.06
3	Gastrula, early	—.164	7.07	32 "	8.74
5	Gastrula, later	—.191	7.06	49 "	7.72
5	Bipinnaria	—.191	7.24	4 to 5 days	8.08
2	Bipinnaria	—.164	7.68	13 to 14 days	9.96

of the egg. This corresponds with the high rate of O_2 consumption in the unfertilized egg and the slight change in the rate upon fertilization (Warburg, 1910 and Loeb, 1913).

3. In *Chaetopterus,* as shown in Table 3, the unfertilized egg has a high redox potential, while that of the sperm is lower. After fertilization, the potential decreases through the trochophore stage which was the last stage measured. This decrease again corresponds with

TABLE 3

SUMMARY OF RESULTS WITH *Chaetopterus*
EXPERIMENTS WITH *Chaetopterus pergamentaceus*

Column 1, the number of experiments at each stage of development (Column 2); Column 3, the E_h or electrode potential in millivolts; Column 4, p_H; Column 5, time after fertilization; Column 6, r_H or redox potential.

1	2	3	4	5	6
No. of experiments	Stage of development	E_h in millivolts	P_H	Time after fertilization	$r_H = 2\ p_H + \frac{E_h}{.03}$
2	Unfertilized eggs	—.161	7.74		10.18
3	Sperm	—.142	7.21		9.72
2	Fertilized eggs	—.177	7.77	15 minutes	9.64
3	4-cells	—.203	7.82	2 hours	8.94
2	Many cells	—.215	7.18	3½ "	7.36

the decrease in rate of O_2 consumption after fertilization as found by Whitaker, 1933.

In short, the evidence points to the conclusion that, if the potential is too low for development, the sperm raises it. If it is too high, the sperm lowers it. There seems to be a critical level necessary for development. Whether this critical level occurs within a definite range of potential for all species alike, cannot be stated from the experiments of only three species of animals. Figure 1 shows the possibility however, that conformity to this potential for the ability to become fertilized, may be the case.

Since the rate of O_2 consumption in marine eggs fertilized by sperm is essentially the same as that when eggs are fertilized artificially, according to the experiments of Warburg, 1910 and Brachet, 1938, the redox potential of certain solutions producing artificial parthenogenesis was measured. In Table 4 measurements were made of these

TABLE 4

REDOX POTENTIAL OF SOME SOLUTIONS PRODUCING ARTIFICIAL PARTHENOGENESIS*

List of solutions producing artificial parthenogenesis (Column 1); Column 2, E_h or electrode potential in millivolts; Column 3, p_H; Column 4, r_H or redox potential.

1	2	3	4
Solution	E_h	P_H	r_H†
50 cc. sea water 10 cc. 2½ M NaCl	+.018	8.0	16.6
50 cc. sea water 7 cc. 2½ M $CaCl_2$	+.074	6.2	14.7
50 cc. sea water 7 cc. 2½ M $MgCl_2$	+.081	6.2	15.1
50 cc. sea water 3 cc. .1 M butyric acid	+.182	3.55	13.1
50 cc. sea water 8 cc. 2½ M NaCl 5 cc. 1% KCN	—.196	9.7	12.9
Sea water alone (Carmel Bay, Calif.)	+.033	8.1	17.1

*Combinations of solutions taken from J. Loeb's "Artificial Parthenogenesis and Fertilization".

†r_H symbol according to W. M. Clark's "Studies on Oxidation-Reduction".

$$r_H = 2\ p_H + \frac{E_h}{.03}$$

solutions without eggs. These show a lower r_H value than that of sea water. In Table 5 eggs were placed in such parthenogenetic solutions

TABLE 5

SHOWING THE EFFECTS OF CHANGES IN REDOX POTENTIAL OF SOLUTIONS CONTAINING UNFERTILIZED *Arbacia* EGGS

No.	Solution	E_h	p_H	r_H	Time
(1)	Sea water alone	+.014	7.95	16.3	———
(2)	Sea water + eggs	—.006	7.7	15.2	After 1½ hours
(3)	Art. s.w. + eggs	—.036	6.7	12.2	"
(4)	Hypertonic s.w. + eggs	—.056	7.7	13.5	"
(5)	Hypotonic s.w. + eggs	—.056	7.8	13.7	"
(1)		No appreciable change			After 2 hours
(2)		—.046	7.8	14.1	"
(3)		—.066	6.7	11.2	"
(4)		—.126	7.6	11.0	"
(5)		—.130	7.6	10.9	"
(6)	Fertilizin	—.076	7.2	11.9	

Composition of solutions:
(1) Fresh filtered sea water (Woods Hole) without eggs.
(2) Sea water, 5 cc. plus 1 cc. of eggs.
(3) Artificial sea water consisting of 100 cc. NaCl, 2 cc. KCl, 2 cc. $CaCl_2$, 7.8 cc. $MgCl_2$, 3.8 cc. $MgSO_4$ (.5 M).
(4) Hypertonic sea water consisting of 50 cc. s.w. + 8 cc. NaCl (2½ M).
(5) Hypotonic sea water consisting of 50 cc. s.w. + 50 cc. distilled water.
(6) Fertilizin from *Arbacia* eggs through the kindness of Dr. Tyler.

In each case, 5 cc. of sea water and 1 cc. of eggs were used. Probable error ±4%; average of 10 experiments each.

with the result that the redox potential was lowered still more. It appears, therefore, that the balance between the p_H and E_h in these solutions is important in mobilizing the electron shift of the enzyme systems responsible for fertilization to induce the energy release for this reaction.

DISCUSSION

These experiments interpret the process of fertilization of the egg as due to differences in redox potentials between egg and sperm, and a change in potential induced by the sperm. The fact that eggs can develop without sperm shows that a change in the physical state of the egg is essential. The experiments on artificial parthenogenesis are too well known for further comment, as is also that of Lillie, 1912, who showed that any piece of the spermatozoon can induce fertilization, rather than a specialized region.

Referring to Fig. 1, a high redox potential is essential for fertiliza-

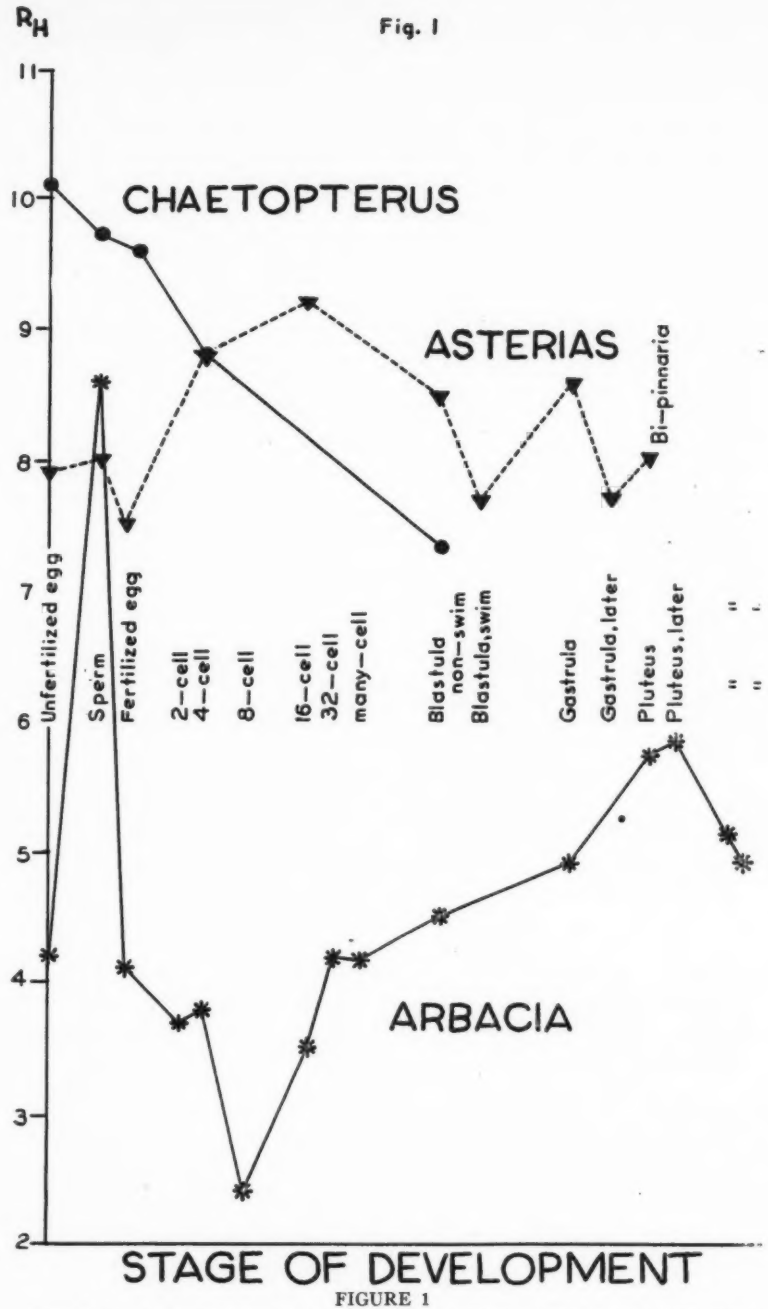

FIGURE 1

Showing the different stages of development of *Arbacia*, *Asterias*, and *Chaetopterus* as abscissae, and the r_H or redox potential as ordinates.

tion, within a certain range. If it is too high, as in *Chaetopterus,* it must be lowered; if too low, it must be raised, as in *Arbacia.* In *Asterias,* the unfertilized egg is already at a high r_H level and requires only a slight change to start development. There is not much difference between the r_H of the unfertilized egg and that of the sperm. This fact fits in with the experiments of others, (Loeb, 1913) showing that these eggs can be very readily fertilized by artificial means. Only a slight impetus is required, such as slightly alkalinized sea water which would reduce the r_H of sea water. Fertilization in this case, also produces very little change in the rate of O_2 consumption, agreeing with the hypothesis that there is only a slight change in the redox potential between the two stages of development. One could infer that the unfertilized *Asterias* egg is in more or less activated state already, requiring only a slight change in r_H to start developing.

As the egg develops, changes in potential again appear. At the first cleavage, the potential is lower. In this stage it is less sensitive to such reagents as cyanide according to Lyon, 1902. Cyanide lowers the potential and one would expect such decrease in sensitivity under these conditions.

No readings could be made immediately after fertilization due to the time required to prepare the materials. So that the first reading is taken at about 20 minutes after fertilization. However the fact that there is such an instantaneous change in the rate of O_2 consumption at fertilization, is indirect evidence that a rise in potential must take place in such eggs as *Arbacia* and subsequent changes in the case of the other species used. It would be of interest if methods could be devised to measure the potential at the moment of fertilization. The decrease in potential after fertilization of the egg can be explained by changes in the metabolism producing such increases in groups representing lowered redox potential. Rapkine, 1930, for example, showed that the —SH group is decreased in concentration after fertilization, but increases again at the first cleavage. These observations, together with that of Lyon, mentioned above, all point to changes in redox potential of the egg as it develops.

Comments on the Fertilization Membrane

It has been shown by different workers that a different set of factors can be used to produce membranes as distinguished from cleavage. Membranes can be produced in the absence of O_2 or in the presence

of KCN as shown by Lillie, 1911, in certain cases. However, for cleavage O_2 must be present and cyanide eliminated. Membrane formation can also be produced by butyric acid or KCN solutions but these reagents cannot be used to produce cleavage. How can we explain these results?

Reference to Table 4 shows that the r_H of sea water is relatively high, but that of butyric acid and of KCN is low. Therefore the lower potentials appear to be effective in membrane formation. Using NaOH in place of KCN would not give the same r_H value as that given by KCN.

When dilute cyanide solutions are used after butyric acid treatment of eggs, as shown by Loeb, 1913, to hasten development, the effect must be one of neutralizing the acid. Table 4 shows that these two solutions have almost identical r_H values, but that butyric acid has a positive E_h value and a low p_H, while cyanide has a negative E_h and a high p_H. The time limit during which the egg must remain in the KCN solution after butyric acid treatment, should give us some facts as to the rate of penetration of the cyanide and the extent of the neutralization.

The effect of cyanide is generally considered as stopping oxidations in cells. It has been shown by the writer, Brooks, 1941, that cyanide lowers the redox potential. If, however, the potential of the cell is already low, there will be no effect by cyanide, depending of course on the concentration used. Such seems to be the case in the unfertilized *Arbacia* egg.

Since oxygen is not essential for membrane formation, and since certain concentrations of KCN will help in its formation, in certain cases, it can be concluded that membrane formation is not associated with oxidations, but incidental to this step in the process of fertilization.

Membrane formation can be considered as a change in the physical state of the proteins. As the potential is changed by fertilization, an alteration in the surface layer of the egg occurs, whether by coagulation or by denaturation or by a change in the state of aggregation of the protein colloids in solution in the egg. A change in the redox potential could account for the formation of such a membrane by creating or destroying valencies. Rapkine, 1930, postulated that cellular division is preceded by a denaturation of the proteins of the sea urchin egg, *Paracentrotus lividus,* and that this denaturation is

accompanied by a splitting of the lipoproteins. The fact that the whole egg undergoes a decrease in viscosity on fertilization, either by sperm or artificially, according to Heilbrunn, 1915, shows that such a theory is consistent with the facts.

When sperm changes the redox potential of the unfertilized egg, according to the writer's hypothesis, the final value results in cleavage. During the process, however, other potentials which are necessarily transitory, as the potential passes through these stages, must be responsible for producing the membrane when a certain level is reached. This can be the explanation for the necessity of using two artificial solutions in certain cases: one for membrane formation and one for cleavage. The fact that the membrane is always formed first, seems therefore to show that it is correlated with cleavage rather than directly related to the process.

Whether the membrane is formed by a change in the pre-existing granules, or by the change in the physical state of the proteins in solution, is not the province of this paper. For such references, see Motomura, 1941, Hollingsworth, 1941, and Harvey and Anderson, 1943. Other changes, such as those of permeability, occur in connection with the fundamental changes in redox potential, but these will not be discussed here.

The Oxidation-Reduction Potential of Sea Water

Sea water is not a solution of pure salts of Na, K, Mg and Ca chlorides and sulphates, but consists also of many inorganic and organic compounds, mostly in dilute concentrations. Among these may be mentioned copper, manganese, arsenic, bromide, iodide and many others. In addition to the metal compounds it contains by-products of the metabolism of many different kinds of plants, bacteria and animals, including the ammonium cycle, the sulphate-sulphide cycle, the nitrate-nitrite cycle and various enzyme systems (Harvey, 1945; Kreps, 1934; Keys, Christensen and Krogh, 1935; Cooper, 1937; Zobell, 1936 and Wachsman and Hotchkiss, 1937). Because these elements are not found in the ocean in like concentrations in different parts of the world, the redox potential will vary within certain limits in different localities. This has been shown in the two localities represented in this paper in Tables 4 and 5.

When the sea water is made hypertonic as shown in Tables 4 and 5 by adding a more concentrated solution of one of the salts of sea

water, the r_H is lowered. When distilled water is added to sea water, the r_H is lowered. It is interesting that these two types of solutions should have r_H values so nearly alike. What the reaction is on the sea water and its constituents is not known, but the facts alone are presented here. It is merely pointed out here that all the solutions in Tables 4 and 5 which have been used to produce artificial fertilization, have a lower redox potential than that of sea water.

Table 5 shows the effects on the r_H value when eggs are added to the solutions. The eggs produce a lowering of the r_H value and this solution is known as "egg water" according to Lillie, 1912, or fertilizin. The r_H of purified fertilizin is also lower than that of sea water, and in the same category as that of the solutions producing artificial parthenogenesis. Kuhn and Wallenfells, 1940, state that fertilizin is made up of echinochrome (a naphthaquinone) which is an oxidation-reduction compound of a low r_H value.

Eggs in sea water or modified sea water lower the p_H value. The common denominator responsible for these changes is still open to hypothesis. For example, shaking or pricking eggs will cause them to develop in some cases (Loeb and Bancroft, 1913). Injury causes an increase in CO_2 production, as is generally known. Loeb has shown that CO_2 will produce artificial parthenogenesis. CO_2 changes the p_H and in this way affects the r_H. Rünnstrom, 1933, has shown that acid formation takes place at fertilization to 20 minutes after, both as a result of fertilization by sperm and by hypertonic or hypotonic solutions. According to Table 5 of the present writing, eggs in parthenogenetic solutions produce a greater change in p_H than in sea water alone, which is best illustrated at the second hour readings. The E_h is also considerably lower in the former case. How would acid formation affect fertilization?

If we take *Arbacia* as an example, acid production by the egg would raise the r_H value of the bathing solution of the egg. The r_H value of the *Arbacia* egg is too low under normal conditions to start development. If the proper r_H value is attained, however, it can begin development. This may be the relation between acid formation and the oxidation-reduction shifts of the enzymes. The exact value appears to be necessary to begin development, and different species have different values.

These experiments answer the question as to why both hypertonic and hypotonic solutions produce parthenogenesis. Eggs placed in such solutions produce a marked lowering of the r_H value of the solution.

Relative Size of Egg and Sperm of *Arbacia* with Reference to the Effect on Redox Potential

It has been objected that the small size of the sperm with reference to the size of the egg (1/50,000 of the volume of the egg) would argue against its ability to change the redox potential of the egg. However, comparisons should be made between the concentrations of *active principle* in egg and sperm, which is reponsible for producing changes, rather than between the whole volume of these cells. At the present writing these values are not known except in one case mentioned below. However, the concentration of "inert matter" in the egg, not influenced by nor influencing directly the redox potential is considerable, if the concentration of cytochrome oxidase in the egg is of the order of 3.5×10^{-5} M as found by Hutchens, Kopac and Krahl (1942). Moreover, Clowes and Krahl (1939) were unable to demonstrate absorption bands of the cytochromes in egg, but found pronounced bands in sperm corresponding to cytochrome a. These few data would indicate that the concentration of the redox enzymes in sperm was at least as large if not greater than that in the egg, and that the relative concentrations of these enzymes is the important factor rather than the over-all size of the egg. Adding to this the fact that sperm has a high rate of O_2 consumption, the evidence points to the conclusion that the enzymes of sperm are sufficiently "activated", i.e., have a high redox potential and are of sufficient concentration to change the ratio of oxidants to reductants in the egg to a higher level.

It is interesting to note here that Hutchens, Kopac and Krahl (1942) found that the location of their "cytochrome oxidase" in *Arbacia* eggs is mainly in the cytoplasmic matrix. This would be of help in a quick reaction of the enzymes of the sperm with the egg. The above workers also found that the concentration of this enzyme was found to be the same in both fertilized and unfertilized eggs. It would therefore appear that the principle effect of adding the sperm was to change the ratio of oxidants to reductants in this state. This difference is seen in the effects of KCN on the two types of eggs as stated earlier in this paper. These facts together with the rise in O_2 consumption on fertilization of *Arbacia* eggs are proof of the change in redox potential at fertilization.

Comments on Cleavage

From Table 4 it can be seen that the redox potential of the hypertonic solutions used to produce cleavage is more positive than that of the solutions producing the fertilization membrane. This agrees with the experiments of others that oxygen must be present and KCN eliminated for cleavage to begin. Sea water alone appears to be too positive in most cases.

When eggs are fertilized by the sperm of other species, such as sea urchins by *Asterias* or *Chaetopterus*, is it probably due to the redox potential of the sperm. Reference to Table 4 shows that the redox potential of *Arbacia* sperm is close to that of *Asterias*.

It seems therefore that it is necessary that the oxidation enzyme systems be raised to a definite potential for cleavage. Since the method used in these experiments did not show this rise in potential at the moment of fertilization, as previously explained, nevertheless, the results show that some such change must occur; the definite link between O_2 consumption and redox potential and the difference in potential between sperm and egg.

At the beginning of the first cleavage or just before this time, the redox potential again becomes low. This fact no doubt, accounts for the observation of Lyon, 1902, that the egg at this stage is helped by certain solutions of KCN or the removal of O_2. This also agrees with the experiments of Rapkine, 1930, who showed that the concentration of the —SH radical changes during development. It is well known that the —SH group is associated with such a system as the cystine-cysteine which is found in most living cells and that the redox potential of this system at p_H 7.0 is very negative.

Changes occur in the potential on further development of the egg. It would be interesting to correlate these changes with other factors, such as the development of enzyme systems.

Conclusions and Summary

The hypothesis upon which these experiments are built is, that *fertilization depends upon the attainment of the correct redox potential of the egg*. There is a difference in potential between egg and sperm. The degree of this difference varies with the species. In some cases it is considerable as in *Arbacia*, in other cases very slight as in *Asterias*.

The electromotively active enzyme systems must be "activated" to produce the beginning of development. This means that the concentrations of oxidants and reductants must be more nearly equalized so that the electron shift can take place at an optimum rate, thereby releasing the necessary energy. In some cases as in *Asterias*, only a slight shift in potential is required. In others as in *Arbacia*, there is a considerable change. This explains why *Arbacia* eggs have a large rise in O_2 consumption on fertilization and *Asterias* does not.

The fact that eggs and sperm contain different proportions of these oxidants and reductants causes them to react.

The formation of acid upon the fertilization of some eggs, whether by sperm or artificially, is an important factor and fits the above theory in that a change in p_H of the egg produces a change in the redox potential.

The increased production of CO_2 or other acids in the egg, caused by such various methods of fertilization as sperm, hypertonic and hypotonic solutions, pricking, shaking or ultra violet light, is assumed by the writer to be the cause of initiating development owing to the changes in redox potential so produced.

When eggs are placed in sea water or modified sea water, they lower the E_h and the p_H. This lowering is more pronounced in modified sea water than in pure sea water.

The redox potential of different species of eggs is different and changes with stages of development.

There is a definite correlation between the rate of O_2 consumption of unfertilized and fertilized eggs and the redox potential of these eggs. Three categories are discussed: when the redox potential of the unfertilized egg is considerably lower than that of sperm, as in *Arbacia*; when it is only slightly lower than that of sperm as in *Asterias*; when it is higher than that of sperm, as in *Chaetopterus*. In the first case, the O_2 consumption rises on fertilization; in the second there is only a slight rise; in the third case, there is a decrease. These three cases illustrate the hypothesis that fertilization of the egg depends upon the correct ratio of oxidants to reductants of the enzyme systems.

Acknowledgments: The technical assistance of Ray F. Garzoli is acknowledged.

References

1. Amberson, W. R. 1928. Influence of O_2 tension upon the respiration of unicellular organisms. *Biol. Bull.*, **55**, 79-91.
2. Barnard, R. D. 1933. The effect of cyanide and a variation in alkalinity on the oxidation-reduction potential of the hemoglobin-methemoglobin system. *Jour. Gen. Physiol.*, **16**, 657-675.
3. Barron, E. S. G. 1930. The catalytic effect of dyes on the oxygen consumption of living cells. *Jour. Gen. Physiol.*, **13**, 483-494.
4. Baumberger, J. P. 1939. The relation between the oxidation-reduction potential and the oxygen consumption of yeast cell suspensions. *Cold Spring Harb. Sympos. VII*, 195-215.
5. Brachet, J. 1938. The oxygen consumption of artificially activated and fertilized *Chaetopterus* eggs. *Biol. Bull.*, **74**, 93-98.
6. Brooks, M. M. 1943. Mechanism of fertilization of eggs. *Federation Proceedings*, **2**, No. 1, 6.
7. ————. 1943. Methylene blue, potassium cyanide and CO as indicators for studying the oxidation-reduction potentials of developing marine eggs. *Biol. Bull.*, **84**, 164-177.
8. Bull, H. B. 1943. Physical Biochemistry. John Wiley & Sons, Inc., New York.
9. Chambers, R., Beck, L. V. & Green, D. E. 1933. Intracellular oxidation-reduction studies. *Jour. Exper. Biol.*, **X**, 142-152.
10. Chambers, R., Pollack, H. & Cohen, B. 1929. Intracellular oxidation studies. *Brit. Jour. Exp. Biol.*, **6**, No. 3, 229-247.
11. Chase, H. Y. 1935. The origin and nature of the fertilization membrane in various marine ova. *Biol. Bull.*, **169**, 159-184.
12. Clark, W. M. 1928. Oxidation-reductions. U. S. Public Health Service Bulletin.
13. ————. 1934. The potential energies of oxidation-reduction systems and their biochemical significance. *Medicine*, **13**, 207-259.
14. Clifton, C. E., Cleary, J. P. & Beard, P. J. 1934. Oxidation-reduction potentials in suspensions of *Escherichia coli*. *Jour. Bact.*, **28**, 541-559.
15. Clowes, G. H. A. & Krahl, M. E. 1939. Studies on cell metabolism and cell division. *Jour. Gen. Physiol.*, **23**, 401-11.
16. Cooper, L. H. N. 1937. Oxidation-reduction potential of sea water. *Jour. Marine Biol. Assoc. Unit. King.*, **22**, 167-176.
17. Genevois, L. 1928. Coloration vitale et respiration. *Protoplasm*, **4**, 67-87.
18. Harvey, E. B. & Anderson, T. F. 1943. The spermatozoon and fertilization membrane of *Arbacia punctulata* as shown by the electron microscope. *Biol. Bull.*, **85**, 151-156.
19. Harvey, W. H. 1945. Recent advances in the chemistry and biology of sea water. Cambridge University Press.
20. Hutchens, J. C., Kopac, M. J. & Krahl, M. E. 1942. Cytochrome oxidase content of centrifugally separated fractions of unfertilized *Arbacia* eggs. *Jour. Cell. and Comp. Physiol.*, **20**, 113-116.
21. Heilbrunn, L. V. 1915. Studies in artificial parthenogenesis. **29**, 149-203.
22. Hollingsworth, J. 1941. Activation of *Cumingia* and *Arbacia* eggs by bivalent cations. *Biol. Bull.*, **81**, 261-276.
23. Keys, A., Christensen, E. & Krogh, A. 1935. The organic metabolism of sea water. *Jour. Mar. Biol. Assoc. U. K.*, **20**, 181.
24. Kitching, J. A. & Moser, F. 1940. Studies on a cortical layer response to stimulating agents in the *Arbacia* egg. *Biol. Bull.*, **78**, 80-91.
25. Kuhn, E. & Wallenfels, K. 1940. Echinochromes as prosthetic groups of high molecular complexes in the eggs of *Arbacia pustulosa*. *Ber.*, **73B**, 458-64.

26. KREPS, E. 1934. Organic catalysts or enzymes in sea water. James Johnstone Memorial Volume. 193-202. Liverpool.

27. KRAHL, M. E., KELTCH, A. K., NEUBECK, C. E. & CLOWES, G. H. A. 1941. Cytochrome oxidase activity in the eggs of *Arbacia* punctulata. *Jour. Gen. Physiol.*, **24**, 597-617.

28. LILIE, F. R. 1912. Studies in fertilization. *Jour. Exp. Zool.*, **12**, 413. *Science*, **36**, 527-530.

29. LILLIE, R. S. 1911. The physiology of cell division. *Jour. Morph.*, **22**, 695-730.

30. LOEB, J. 1913. Artificial parthenogenesis and fertilization. Chicago: The University of Chicago Press.

31. LOEB, J. & BANCROFT, W. 1913. Sex of parthenogenetic tadpole and frog. *Jour. Exp. Zool.*, **14**, 275-277.

32. LYON, E. P. 1902. Effects of KCN and of lack of O_2 upon the fertilized eggs and embryos of the sea urchin (*Arbacia punctulata*). *Amer. Jour. Physiol.*, **7**, 56-75.

33. MOORE, A. R. 1930. Fertilization and development without membrane formation in the egg of *Dendraster excentricus*. *Protoplasma*, **9**, 18-24.

34. MOTOMURA, I. 1941. Materials of the fertilization membrane in the egg of echinoderms. *Tohoku Imp. Univ. Reports*, **16**, 345-363.

35. MOSER, F. 1940. Cortical layer response to stimulating agents in the *Arbacia* egg. *Biol. Bull.*, **78**, 68-79.

36. RAPKINE, L. 1930. Sur les processus chemiques au cours de la division cellulaire. *Comp. rend. Acad. Sci.*, **191**, 871-874.

37. RAPKINE, L. & WURMSER, R. 1926. On intracellular oxidation-reduction potential. *Proc. Roy. Soc. London*, B, **102**, 527-530.

38. RUNNSTROM, J. 1933. Zur Kentniss der Stoffwechsel vorgange bei den Entwicklungserregung des Seeigeleies. *Biochem. Zeit.*, **258**, 257-279

39. ———. 1935. Influence of iodoacetate on activation and development of the eggs of *Arbacia punctulata*. *Biol. Bull.*, **69**, 351-355.

40. WACHSMAN, S. A. & HOTCHKISS, M. 1937. Viability of bacteria in sea water. *Jour. Bact.*, **33**, 389.

41. WARBURG, O. 1908. Beobachtungen über die Oxydations prozesse in Seeigelei. *Zeit. Physiol. Chem.*, **57**, 1-16.

42. ———. 1910. Über die Oxydation in lebenden Zellen nach Versuchen am Seeigelei. *Zeit. f. Physiol. Chem.*, **66**, 305-340.

43. WHITAKER, D. M. 1933. The rate of O_2 consumption by fertilized and unfertilized eggs. *Jour. Gen. Physiol.*, **16**, 497-528.

44. ZOBELL, C. E. 1935. Oxidation-reduction potentials and the activity of marine nitrifiers. *Jour. Bact.*, **29**, 78.

Acceptable work will be published within three months with no restrictions as to length. To accomplish this, papers must be accompanied by a minimum order for 200 reprints at $3.00 per page of text. Tables, illustrations, and mathematical equations approximately $4.50 per page. Rates for larger orders of reprints will be supplied on request.*

All papers must be accompanied by an abstract not exceeding 225 words, for *Biological Abstracts*, and by a running title not in excess of 35 letters. Papers may be written in English, French, German, or Italian.

References should be arranged at the end of the paper in alphabetical order by author, and then numbered. The title must always be given, and the inclusive pages indicated. Following is a correct sample form of journal reference:

12. Doe, J. 1942. The preparation of manuscripts. *Growth*, **6**, 700-725.

If a book, the above sample reference form would be:

12. Doe, J. 1942. The Preparation of Manuscripts. New York: Holt. Pp. 400.

In the text, references should be made to the author and either the year or reference number, e.g., Liebig and Wöhler (1837) or Liebig and Wöhler (12),—or still more exactly, Liebig and Wöhler (12, p. 15). Summary and conclusions should be included; footnotes should be avoided. Changes from copy will be charged to the author.

The Auxiliary Publication Service of the American Documentation Institute at Washington is available for the publication, as micro-film or enlarged microphotographs, of excessive tabular or illustrative material. The Institute will also serve as a central deposit for original data.

Manuscripts should be submitted in finished form, ready for print, with the author's name on each table and figure. Manuscripts should be typewritten in double or triple spacing, on one side of the paper. Carbon copies cannot be accepted. Illustrations should not exceed 8½×11 inches; they should be numbered and the desired reduction should be indicated. They should be accompanied by complete legends, and their correct place in the manuscript should be indicated. For various methods of illustrating, authors are recommended to consult the Wistar Institute *Style Brief*.

*The Editors regret that, due to increased publication costs, it will be necessary to increase charges temporarily by 15 per cent.

TABLE OF CONTENTS

Paper		Page
304	Action de la lumiére et de la temperature sur la croissance et la sexualité des males et des femelles de *Mercurialis annua* L By Georges Troupin	343
305	Tolerance of larvae of Drosophila for amino acids: Methionine, Cystine, and Cysteine By Louise Palmer Wilson	361
306	The combined effect of colchicine and X-rays on the growth of Allium cepa seeds By A. Back	375
307	Nitrate and Phosphate vs. 1-Proline as factors in the behavior of *Ulva* By Philip R. White	381
308	The mechanism of fertilization of eggs By Matilda Moldenhauer Brooks	391

GROWTH SYMPOSIA

Proceedings of the annual meeting of the Society for Growth and Development presented as the Sixth Growth Symposium at Kingston, Rhode Island, August, 1946, will appear at an early date as a supplement to GROWTH, volume 10.

Copies of the earlier symposia are still available. *First Symposium* (1939) $2.00 (for members of the Society for Growth and Development, $1.00). *Second Symposium* (1940), *Third Symposium* (1941), *Fourth Symposium* (1942), $3.00 (for members, $2.00).

All orders through *The Editors of Growth, Lankenau Hospital Research Institute, Girard and Corinthian Avenues, Philadelphia 30, Pennsylvania.*